The Contribution of *Acinetobacter baumannii* A424 resistance island Tn*AbaR23* on fitness and virulence associated phenotypes

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Abstract

The Contribution of *Acinetobacter baumannii* A424 resistance island Tn*AbaR23* on fitness and virulence associated phenotypes

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The *Acinetobacter baumannii* specific resistance islands called AbaR islands are foreign acquired resistance islands and are widely represented in the genome of multi-drug resistance [MDR] *A. baumannii* strains. The resistance genes and determinants within AbaR are found to contribute minimally towards the overall MDR phenotype of the host. Therefore, the maintenance of AbaR islands purely for the enhancement and development of antimicrobial resistance phenotype appears to be an inadequate explanation. This study investigates the contribution of AbaR island called Tn*AbaR23*, in a MDR *A. baumannii* strain A424, towards the resistance and virulence phenotypes of the host. The Tn*AbaR23* deleted mutants were created by allelic exchange and the fitness and virulence of mutants was compared with their parent in a head-to-head competition assay and *Galleria mellonella* killing assay. During this study, spontaneous deletion of internal region of Tn*AbaR23* was observed in wild type A424 sub-population. In this study, the role of transposase gene *tnpA* in the spontaneous deletion within Tn*AbaR23* was also investigated. The spontaneous mutants of A424 were isolated and analysed for their antimicrobial resistance, fitness and virulence phenotypes. Although complete or partial deletion of Tn*AbaR23* had no effect on the growth of bacteria, the wild type appeared less fit in a head-to-head growth competition with Tn*AbaR23* deleted and spontaneous mutants. Except from the anticipated antibiotic susceptibility upon the deletion of Tn*AbaR23*, the overall resistance phenotype in mutants remained unchanged. Intriguingly, the Tn*AbaR23* deleted and spontaneous mutants exhibited reduced virulence compared to their parent in a *G. mellonella* killing assay. Despite the associated cost, the maintenance of Tn*AbaR23* appears to be vital to exhibit enhanced virulence or pathogenesis in *A. baumannii* strain A424. It is therefore possible that *A. baumannii* AbaR islands positively contribute towards the development of traits that are vital for the survival of these bacteria.

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Abbreviations

Abbreviation	Explanation
%	Per cent
λ	lambda
µg	microgram
µl	microliter
°C	degree Celsius
Blast	Basic local alignment search tool
bp	base pairs
DNA	deoxyribose nucleic acid
dNTP	dinucleotide triphosphate
Etest	Epsilometer test
h	hours
kb	kilobase pair(s)
Mb	Megabase pairs
MDR	Multi-drug resistant
min	Minutes
mM	milli molar
mm	Millimetre
ng	nanograms
nm	nanometer
OD	Optical Density
PDR	Pan-drug resistant
rpm	Revolutions per minute
s	seconds
sp.	Species
spp.	Plural of species
UV	Ultra violet
WT	Wild type
w/v	Weight/volume
XDR	Extensive-drug resistant

Presentations

Fifth Annual Postgraduate Conference

University of Leicester, Department of Infection, Immunity and Inflammation, April 2013

Oral presentation entitled: "Contribution of Tn*AbaR23* on the phenotype of *Acinetobacter baumannii"*

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Oral presentation entitled: "Contribution of Tn*AbaR23* island on the phenotype of *Acinetobacter baumannii* strain A424"

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Poster presentation entitled: "Fitness and virulence-associated phenotypes of the plastic Tn*AbaR23 Acinetobacter baumannii* A424 resistance transposon"

2nd London Postgraduate Research Symposium on Bacterial Pathogenesis and Host Response 2015

Birkbeck, University of London, Friday 27th November 2015

Oral presentation entitled: "*Acinetobacter baumannii* resistance island and its contribution on the fitness and virulence associated phenotypes"

1 Introduction

1.1 *Acinetobacter*

The word *Acinetobacter* is originally derived from Greek word *akinetos* meaning unable to move. The genus *Acinetobacter* belongs to non-motile, free-living, non-fermentative, oxidase-negative, strictly aerobic gram negative bacteria (Brisou and Prevot 1954). These bacteria can utilize a wide range of carbon sources and can be easily grown on nutrient agar in the laboratory at 20°C -30°C (Bergogne-Berezin and Towner 1996). These bacteria are also known to be intrinsically resistant to many antimicrobial agents. In recent years, *Acinetobacter baumannii* has been frequently associated with serious nosocomial and community associated infections. Pathogenic strains of *A. baumannii* are considered opportunistic as they often cause serious and life threatening infections in immunosuppressed patients (Falagas *et al.* 2006). In recent decade, there has been a global increase in the reports of isolation of multi drug-resistant (MDR), pan drug-resistant (PDR) and extensively drug-resistant (XDR) *A. baumannii* strains from various clinical settings (Gottig *et al.* 2014, Teo *et al.* 2015). In the absence of new drug as a treatment alternative, the option for managing *A. baumannii* infections is becoming highly problematic and restricted.

The following sections will describe the taxonomy, ecology and clinical importance of *A. baumannii*. The factors associated with drug resistance in *A. baumannii* will also be briefly discussed in the sections below paying particular attention to the *A. baumannii* specific genomic resistance islands called ***A****cinetobacter* ***ba****umannii* **R**esistance island or AbaR in short followed by sections providing a synopsis of evolution and divergence of AbaR together with the discussion on the AbaR associated resistance phenotype in *A. baumannii*. The final section will discuss in detail the aims and objectives of the study.

1.1.1 Taxonomy of genus *Acinetobacter*

Acinetobacter belongs to the family Moraxellaceae, order Gamma proteobacteria, and genus *Acinetobacter* (Peleg *et al.* 2008). There are currently 40 validly named species of *Acinetobacter* [database assessed until September 2015] with 13 species named waiting authentication (Table 1.1).

Table 1.1 *Acinetobacter* species isolated from various sources

Species	Isolation from	Reference
Acinetobacter antiviralis	Tobacco plant root	(Lee *et al.* 2009) name not validly published
Acinetobacter apis	Honey bee	(Kim *et al.* 2014) name not validly published
Acinetobacter bouvetii	activated sludge	(Carr *et al.* 2003)
Acinetobacter. baylyi	activated sludge	(Carr *et al.* 2003)
Acinetobacter bohemicus	Soil and water ecosystem	(Krizova *et al.* 2014), name effective but not validly published
Acinetobacter baumannii	Soil, water, natural reservoir unknown	(Bouvet 1986)
Acinetobacter boissieri	Floral nectar	(Alvarez-Perez *et al.* 2013)
Acinetobacter brisouii	wetland	(Gerner-Smidt *et al.* 1991)
Acinetobacter bereziniae	soil	(Bouvet 1986)
Acinetobacter beijerinckii	Human specimen	(Nemec *et al.* 2009)
Acinetobacter calcoaceticus/baumannii complex	Clinical isolate	(Gerner-Smidt *et al.* 1991)
*Acinetobacter pittii (*formerly genomic species 3*)*	Water, soil, vegetable, human skin	(Nemec *et al.* 2011)
Acinetobacter genomospecies 13	Clinical isolate	Tjernberg and Ursing 1989)
Acinetobacter gandensis	Horse and cattle	(Smet *et al.* 2014)
Acinetobacter genomosp. 13BJ	Clinical isolate	(Tjernberg and Ursing 1989)
*Acinetobacter nosocomialis (*formerly genomic species 13TU*)*	Clinical isolate	(Nemec *et al.* 2011)
Acinetobacter genomosp. 14BJ	Clinical isolate	(Bouvet and Jeanjean 1989)
Acinetobacter genomosp. 15BJ	Clinical isolate	(Bouvet and Jeanjean 1989)
Acinetobacter genomosp. 15TU	Clinical isolate	(Tjernberg and Ursing 1989)
Acinetobacter genomosp. 16BJ	Clinical isolate	(Bouvet and Jeanjean 1989)
Acinetobacter genomosp. NB14	Not known	Espinal P *et al.* (unpublished 2014)
Acinetobacter glutaminasificans	Soil	(Roberts *et al.* 1972) name not validly published
Acinetobacter guangdongensis	Lead-zinc ore	(Feng *et al.* 2014)
Acinetobacter guillouiae	Water, soil, vegetable, human intestinal tract	(Nemec *et al.* 2010)
Acinetobacter gyllenbergii	Human specimen	(Nemec *et al.* 2009)

Species	Isolation from	Reference
Acinetobacter gerneri	Activated sludge	(Carr *et al.* 2003)
Acinetobacter harbinensis	River water	(W. Li *et al.* 2014)
Acinetobacter haemolyticus	Clinical isolate	(Bouvet 1986)
Acinetobacter indicus	hexachlorocyclohexane dump site	(Malhotra *et al.* 2012)
Acinetobacter johnsonii	Water, soil, human skin, human faeces	(Bouvet 1986)
Acinetobacter junii	human skin , mucous membrane, activated sludge	(Bouvet 1986, Carr *et al.* 2003, Vaneechoutte *et al.* 2008)
Acinetobacter kookii	soil	(J. Y. Choi *et al.* 2013)
Acinetobacter kyonggiensis	Sewage treatment plant	(Lee and Lee 2010) name effective but not validly published
Acinetobacter lwoffii	human skin and mucous membrane	(Bouvet and Jeanjean 1989)
Acinetobacter marinus	Sea water	(Yoon *et al.* 2007) name not validly published
Acinetobacter nectaris	Floral nectar	(Alvarez-Perez *et al.* 2013)
Acinetobacter oleivorans	Diesel-soil	(Kang *et al.* 2011) name effective but not validly published
Acinetobacter oryzae	Wild rice	(Chaudhary *et al.* 2012) name effective but not validly published
Acinetobacter pakistanensis	Waste water treatment pond	(Abbas 2014) name effective but not validly published
Acinetobacter parvus	human and dog samples	(Nemec *et al.* 2003)
Acinetobacter psychrotolerans	hydrocarbon	Yamihara K *et al.* (unpublished 2005)
Acinetobacter puyangenesisi	Canker bark	(Li *et al.* 2013)
Acinetobacter qingfengensis	Canker bark	(Y. Li *et al.* 2014)
Acinetobacter rudis	Raw milk and raw waste water	(Vaz-Moreira *et al.* 2011)
Acinetobacter radioresistens	Cotton and soil	(Nishimura Y 1988)
Acinetobacter soli	Forest soil	(Kim *et al.* 2008)
Acinetobacter seohaensis	Sea water	(Yoon *et al.* 2007) name not validly published
Acinetobacter seiffertii	Human clinical specimen	(Nemec *et al.* 2015)
Acinetobacter schindleri	human specimen	(Nemec *et al.* 2001)
Acinetobacter tjernbergiae	activated sludge	(Carr *et al.* 2003)
Acinetobacter towneri	activated sludge	(Carr *et al.* 2003)
Acinetobacter tandoii	activated sludge	(Carr *et al.* 2003)
Acinetobacter ursingii	Clinical isolate	(Nemec *et al.* 2001)
Acinetobacter venetianus	lagoon	(Di Cello *et al.* 1997)
Acinetobacter variabilis	human and animal	(Krizova *et al.* 2015)
Acinetobacter xiameninsis	Intestinal content of flounder	Lai Q and Shao Z (unpublished 2006)

1.1.2 Cell Structure and metabolism of *Acinetobacter*

Acinetobacter are rod shaped bacteria that often occur in pairs and tend to become coccoid towards the stationary growth phase. These bacteria range from 1.0 - 2.5 µm and form a pale yellow colony on nutrient agar media. These bacteria can utilize a wide range of carbon sources except glucose (Baumann 1968). *Acinetobacter* can be isolated from the clinical samples on the commercially available selective medium such as Herellea agar or Difco (Charles 1968) or CHROMagar™ where the colonies of *Acinetobacter* sp. appear red. Specific antibiotics can be supplemented in the selective medium to suppress the non-specific bacteria. Other selective media such as Leeds *Acinetobacter* medium, minimum medium supplemented with acetate and 0.2 % sodium acetate agar in a mineral salt base has been used to isolate *Acinetobacter* sp. from various samples (Berlau *et al.* 1999). Several molecular methods have been developed to distinguish *Acinetobacter* at species level. The common molecular methods frequently used to distinguish *Acinetobacter* sp. include 16S rRNA gene restriction analysis (ARDRA), amplified fragment length polymorphism (AFLP), ribotyping, tRNA spacer fingerprinting, restriction analysis of 16S-23S rRNA, sequence analysis of *rpoB* and flanking region, detection of $bla_{\text{OXA-51}}$, and multi-locus sequence typing (MLST). Despite its inconvenience, the DNA-DNA hybridization technique remains a reference method to identify *Acinetobacter* at species level (Peleg *et al.* 2008).

1.1.3 Ecology and epidemiology of *Acinetobacter*

Species of *Acinetobacter* are ubiquitous and these bacteria have been isolated from a wide variety of surfaces including food, vegetables, environmental samples like soil and hospital surfaces including bed rails and pillows (Table 1.1). *Acinetobacter* often show minimum nutrient requirement because of which they can survive on various clinical surfaces and can endure harsh environmental conditions for a prolonged period of time (Baumann *et al.* 1968, Costerton 1999). Despite being considered as a

common skin commensal, these bacteria have been isolated as deadly pathogens from combat victims and victims of natural disasters (CDC 2004, Lockhart *et al.* 2007).

Various species of *Acinetobacter* are usually non-pathogenic to healthy individuals but *A. baumannii* colonization can cause potentially lethal infections especially in patients with compromised immune system (Maragakis and Perl 2008). In the early 1960s, species of *Acinetobacter* were considered as low-grade pathogens and they were usually ignored when isolated from clinical samples (Kempf and Rolain 2012). In the last few decades, and especially after the increased incidence of *A. baumannii* colonization associated with combat victims returning from the Iraq conflict, this bacteria has gained reputation as a notorious opportunistic pathogen among microbiologists and clinicians (Lockhart *et al.* 2007).

Among various species of *Acinetobacter, A. baumannii* are clinically important because they belong to an epidemic clonal group EC I and EC II and are frequently associated with hospital associated and community-acquired infections (Post *et al.* 2010). The reports of *A. baumannii* infections in trauma patients and patients from natural disasters in the community are increasing rapidly. In the clinical settings, colonization of *A. baumannii* strains in a wide range of surfaces and devices and can cause various infections like ventilator associated pneumonia, skin and wound infections, bacteraemia leading to sepsis and septic shock, urinary tract infections, meningitis, surgical site infections, abdominal infections, central nervous system infections (Gaynes and Edwards 2005). Studies have indicated that presence of invasive medical devices, serious underlying diseases, previous history of receiving broad-spectrum antibiotics, major surgery, burns, immune-suppression, chronic alcoholism, cancer, bronchopulmonary disease etc. can be a predisposition factors for *A. baumannii* infections (Kempf and Rolain 2012).

Successful treatment of the infection relies on the resistance status of the causative agent. Despite the best efforts to contain and combat the *A. baumannii* associated infections, the report of MDR, PDR and XDR *A. baumannii isolates* colonizing various community and healthcare settings around the globe is increasing in an alarming rate

(Jung and Park 2015). Due to this rapid emergence and dissemination of antibiotic resistant in *A. baumannii* and no new drugs on the development pipeline, the antibiotic options for the treatment of *A. baumannii* infections is limited (Ozdem *et al.* 2011) and the treatment of infections has become highly problematic and restricted. The arsenal of antibiotics currently in use in the frontline of *A. baumannii* infection includes colistins, sulbactams co-formulated with ampicillins, tigecycline, minocycline, carbapenems, piperacillin/tazobactam, cefepime, doxycycline, aminoglycosides and quinolones (Dijkshoorn *et al.* 2007, Viehman *et al.* 2014). Due to the sub-optimal pharmacokinetics of the drugs and rapid emergence of resistance, combination drug therapy for infection treatment is attracting frequent attention (Batirel *et al.* 2014).

1.1.4 Resistance and virulence determinants in *A. baumannii*

Bacterial are also well known for their ability to adopt various resistance mechanisms in order to resist the antimicrobial agents present in their environment. Enzymatic inactivation of the antimicrobial agent and extruding the toxic agents by efflux are widespread in bacteria as a mechanism of antimicrobial resistance. Bacteria can also reduce the entry of antimicrobial agents into the cells by reducing the number of antimicrobial targets or protecting the antimicrobial targets (like porin) on the cell surface. Replacement of the susceptible antimicrobial targets and acquisition of novel pumps to extrude antimicrobial agents from cell are also common resistance mechanisms in bacteria (Andersson and Hughes 2010).

In a complex natural ecosystem, bacteria evolve and thrive either by mutating their intrinsic or acquired genes or by acquiring foreign DNA that carry beneficial genes (van Hoek *et al.* 2011). Mobile Genetic Elements (MGE) like plasmids, transposons, insertion sequences and integrative conjugative elements that carry beneficial genes are favourably taken up by bacteria, by means of transformation, conjugation or transduction, to enhance the resistance and virulence phenotypes (Andersson and Hughes 2010, Domingues *et al.* 2012). The successful acquisition and integration of MGEs and foreign genetic elements that carries advantageous traits is believed to an

important factor in the evolution of bacterial genome (Roberts and Kreth 2014, Mullany *et al.* 2015).

A. baumannii displays a combination of features like survival under harsh condition, efficient host adherence and invasion, antibiotic resistance and ability to acquire foreign genetic elements that has enabled this bacterium to emerge, spread and persist as a successful pathogen (Frost *et al.* 2005, Smith *et al.* 2007).

The core genome of *A. baumannii* contains a range of genes and operons that confers innate resistance against a wide variety of antimicrobial agents. *A. baumannii* show high level of intrinsic antimicrobial resistance by means of regulation of antibiotic resistance genes for the production of Aminoglycoside Modification Enzymes (AMEs), *Acinetobacter*-derived cephalosporinases (AmpC β-lactamase), other β-lactamases coded by $bla_{OXA\text{-}51\text{-}like}$ genes, extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs). These bacteria exhibit sulbactam resistance by reducing the expression of penicillin binding protein 2, a drug target for sulbactams, on the surface of their cells (Viehman *et al.* 2014). Modification of the target protein for colistin and fluoroquinolones is also a well-known mechanism of resistance in these bacteria. These bacteria also limit the entry of antimicrobial agents into the cells by down regulating the expression of porin channels and mutating the recognition sites for antimicrobial agents like outer membrane protein (Hamidian *et al.* 2013).

In *A. baumannii*, various efflux pumps are actively involved in extruding antimicrobial agents like tetracycline, rifampicin, fluoroquinolones that had gained access into the cell. Efflux pumps belonging to the Major Facilitator Super-family (MFS) like tetracycline repressor protein class A and class B (TetA/B), chloramphenicol efflux protein (CmlA), the Resistance-Nodulation-Division super-family (RND) like AdeABC and the Multidrug And Toxic compound Extrusion family, MATE, like AbeM have been well studied for their function and substrate in various *A. baumannii* strains (Vila *et al.* 2007).

Sequence analysis of *A. baumannii* have also shown the presence of various inherent and acquired genes and determinants that are likely to be associated with the virulence of this bacteria. Some of the determinants associated with the virulence of *A. baumannii* include CsuA/BABCDE chaperone-usher pili assembly system, siderophores, type I pili, hemin utilization proteins, outer membrane protein OmpA, *Aba*I auto inducer synthase, biofilm associated protein Bap, two-component regulatory system BfmRS, penicillin binding protein, PNAG-constituted biofilm, capsule, lipopolysaccharide, phospholipase D and phospholipase C (Cerqueira and Peleg 2011).

A swift genetic response to the changing environmental condition by means of regulation of native genes and uptake of beneficial genes from the environment is crucial for the survival and persistence of the bacteria (Frost *et al.* 2005, Poirel and Nordmann 2006, Yoon *et al.* 2013). Genome analysis shows that *A. baumannii* has acquired a number of resistance determinants including transposons, plasmids, genomic islands (GIs), insertion sequences (IS), and integrons from its environment by horizontal gene transfer (Diancourt *et al.* 2010, Imperi *et al.* 2011). Various species of *Acinetobacter* possess the ability of naturally acquiring assimilating and disseminating mobile genetic elements by means of horizontal gene transfer (Metzgar *et al.* 2004); however, there is no report on natural transformation exhibited by *A. baumannii* strains. This thesis report *A. baumannii* specific resistance island AbaR and its role in various phenotypes of the host strain. The following sections provide a synopsis on the MGEs focussing particularly on the *A. baumannii* specific genomic island.

1.2 Mobile Genetic Elements (MGEs) in *A. baumannii*

The average size of *A. baumannii* genome is about 4 MB. The pan genome of *A. baumannii,* which corresponds to a sum of all core (present in all strains of a species) and dispensable genome (absent in at least one strain), contains 8818 genes of which less than 17 % of the genes are considered as core-conserved genome and ~ 83 % as dispensable genome (Field *et al.* 2006, Imperi *et al.* 2011). Of this vast pool of dispensable genome, 25 % of the genes are again found to be unique and strain

specific (Imperi *et al.* 2011). A major portion of this so called dispensable genome comprises of genomic islands (GIs). Some *A. baumannii* GIs are strain specific while others are completely or partially conserved in more than one strain (Di Nocera *et al.* 2011). The nomenclature of these GIs is based on the nature of genes they carry, like resistance islands (carry resistance determinants), metabolic islands (carry genes for specific metabolisms) and phase islands (carry genes coding phase products).

The genome sequence analysis shows that almost all of the resistance islands in *A. baumannii* are a composite of several mobile genetic elements (MGEs) like transposons and insertions sequences. The MGEs are DNA that are able to move between the cells during horizontal gene transfer via plasmids, bacteriophages, Insertion Sequence (IS) elements, Integrative and Conjugative Elements (ICEs), transposons (Tns) and miniature inverted repeat transposable elements (MITEs). Interestingly, the origin of *A. baumannii* resistance island-associated MGEs has been traced to other bacterial species including *Pseudomonas*, *Salmonella* and *E. coli*. (Fournier *et al.* 2006). In the following sections, *A. baumannii* specific resistance islands called AbaR will be discussed and the similarities of AbaR with bacterial transposon Tn*7* will also be highlighted.

1.2.1 *Acinetobacter baumannii* Resistance (AbaR) island

During a comparative genome study of MDR *A. baumannii* strain AYE and susceptible strain SDF (Fournier *et al.* 2006), a cluster of around 45 antimicrobial resistance determinants were found as a resistance island in 86 kb island on the chromosome of AYE. The comparative genome analysis also showed the presence of similar island inserted in the identical position in the chromosome of strain SDF, however, this island in the SDF strain was much smaller and it was empty, in a sense, the island was lacking determinants for antimicrobial resistance. Since the AbaR in AYE was the first island of this nature to be identified in *A. baumannii*, it was named *Acinetobacter baumannii* resistance island 1 or AbaR1. To-date, various AbaR-like islands have been reported from numerous *A. baumannii* strains and they are numbered from AbaR0 to AbaR27

(Kochar *et al.* 2012, Zhu *et al.* 2013). Undoubtedly, several AbaR islands are waiting to be identified as the sequence data of *A. baumannii* becomes available. AbaR islands come in various sizes, the largest described so far is AbaR26 from carbapenem resistant MDR strain BJABO7104 , a clinical isolate from Beijing hospital (Zhu *et al.* 2013), and the smallest being AbaR0 in strain ATCC17978, associated with fatal meningitis (Smith *et al.* 2007). More recently, the AbaR islands which were originally believed to be specific to *A. baumannii*, have been reported in non-*baumannii* species of *Acinetobacter* as a result of possible interspecies AbaR transfer (Kim and Ko 2015).

Towards the 5' end, AbaR islands carry an array of five transposase genes viz. *tniA*, *tniB*, *tniC*, *tniD*, *tniE* that are predicted to be essential for transposition. The AbaR islands are bracketed by 19/26 bp imperfect inverted repeat (IR) sequences and their insertion at the competence related chromosomal gene *comM* results in a perfect duplication of five nucleotides ACCGC. Since most of the AbaR islands are known to occupy chromosomal *comM* site, the *com*M gene in *A. baumannii* is often described as a hotspot where AbaR islands integrate in a site and orientation specific manner (Adams *et al.* 2008). The AbaR islands are also known to occupy non-*com*M sites and in some strains more than one type of AbaR islands are found integrated within their genome (Smith *et al.* 2007, Adams *et al.* 2008, Adams *et al.* 2010, Rose 2010).

1.2.2 AbaR islands distantly related to Tn*7* transposon

Transposons are MGEs and possess the ability to move (transpose) or hop from one location to another within a genome. Transposons use their own recombinase enzyme (called transposase) to recombine at a target regardless of sequence homology (Joseph and Craig 2001). These mobile elements are central to the genome evolution and diversity because of their role in gene expression, recombination, and genetic modulation and above all, their ability to move by vertical or horizontal gene transfer (Frost *et al.* 2005). There are two kinds of transposons, copy-and-paste transposons or retrotransposons and cut-and-paste transposons or DNA transposons (Craig 1997). In case of retrotransposons, transposon transcribes itself as RNA that is then reverse

transcribed as a new copy of DNA for transposition. The DNA transposons on the other hand, does not form RNA intermediate and jumps from its original site in its entirety to integrate elsewhere in the genome. Bacterial transposon Tn*7* is a cut-and-paste transposon and five "transposition genes" towards its 5' end tightly regulate the mechanism of transposition. The genes and determinants involved in antimicrobial resistance, DNA repair, DNA modification, DNA restriction, several other transposase including many other genes of unknown functions are present towards the 3' end of Tn*7*.

The five Tn*7* transposition genes code for transposases proteins viz. TnsA, TnsB, TnsC, TnsD, TnsE, and they show a highly conserved synteny. A stretch of 90 bp and 150 bp non-identical segments necessary for the transposition lie at the extreme left and right ends of the Tn*7* and a short 19/26 bp imperfect IR sequence brackets Tn*7* transposon. During the Tn*7* transposition, transposases TnsD and TnsE select the target site *att*Tn*7.* Once TnsD/E binds at the target*, att*Tn*7*, TnsAB excise the DNA from the native site as a double stranded DNA. TnsC forms a DNA complex with the excised transposon and TnsD/E at the target site (Waddell and Craig 1989). The 3' ends attach covalently with the target site but a five bp gap flanks the 5' end. The process of target-site duplication then repairs this staggered cut. This results in five bp (ACCGC) target site duplication, also called direct repeat sequence or DR is a characteristic feature of Tn*7* transpositional recombination (Joseph and Craig 2001). In several bacteria, many Tn*7*-like elements have been identified based on sequence homology with Tn*7* transposition genes.

The AbaR islands are often considered as composite transposons that are built on the "backbone" of other transposons like Tn*6019*, Tn*6021* and Tn*6022* (Post and Hall 2009, Post *et al.* 2010, Seputiene *et al.* 2012). These three common AbaR backbone transposons, Tn*6019*, Tn*6021* and Tn*6022*, are closely related to each other in a sense that they have same gene cluster towards their 5' end and 3' end and their mechanism of transposition is identical (Figure 1.1). The complexity and mosaic internal architecture of AbaR islands is believed to have arisen due to integration of several transposons and IS elements from various bacteria like *E. coli*, *Pseudomonas* spp. and

Salmonella spp. (Fournier *et al.* 2006). In this section, the basic structure of AbaR islands will be explained aiming to provide a synopsis on the diversity of AbaR islands in *A. baumannii* genome.

1.2.3 Features of AbaR island backbone

A majority of AbaR islands are borne on the backbone of an arsenic resistance transposon Tn*6019 (Post et al. 2010)*. In the transposon Tn*6019*, towards the 5' end immediately after the 19 - 26 bp left IR sequence an array of five gene cluster *tniA, tniB, tniC,* orf2 and orf3 (Figure 1.1) is present which is believed to be essential for transposition. This cluster of genes is therefore considered as "core transposition genes" or "transposition machinery" (Post and Hall 2009).

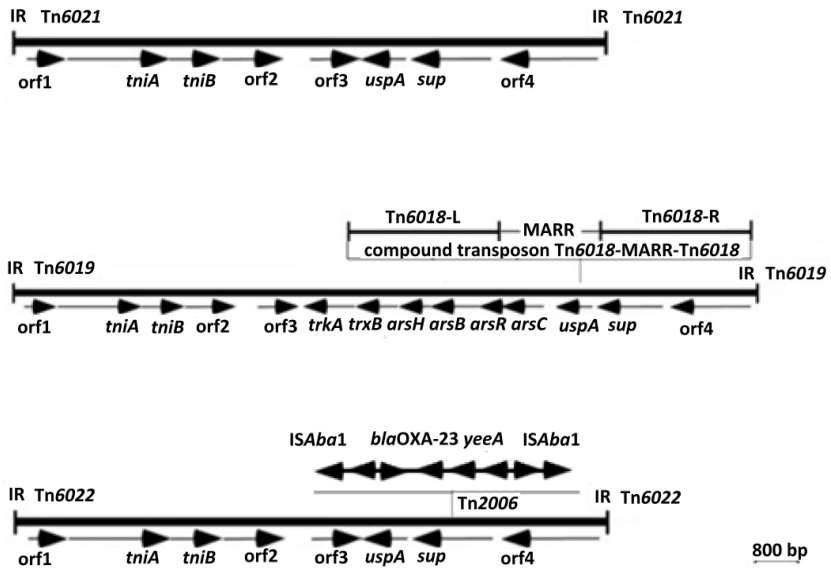

Figure 1.1 Common transposons forming backbone of AbaR islands.

Schematics of Tn*6019* and Tn*6021* are obtained from (Post *et al.* 2010); Tn*6022* was redrawn from Kochar *et al* (2012). The *Tn6021* backbone genes comprise of five transposase genes Orf1, *tniA*, *tniB*, Orf2 and Orf3 including universal stress protein coding gene *uspA*, sulfate permease gene *sup* and Orf4. In Tn*6019*, the *uspA* gene is interrupted by compound transposon made of multiple antibiotic resistance region MARR enclosed between two copies of directly oriented Tn*6018.* The sulfate permease gene *sup* is interrupted by Tn*2006* in AbaR elements carrying Tn*6022* in their backbone. MARR region being very large is not drawn to the scale.

Towards the 3' end of this transposon are three genes, universal stress protein *uspA, orf4* and sulfate permease *sup.* The *uspA* gene, which belongs to the universal stress protein family and whose expression is enhanced under stressful conditions, is usually interrupted by a "compound transposon" Tn*6018*-MARR-Tn*6018*.

The Tn*6018* is a heavy metal transposon and two directly oriented copies of this transposon enclose a **M**ultiple-**a**ntibiotic **r**esistance **r**egion (MARR) to make Tn*6018*-MARR-Tn*6018* compound transposon (Post and Hall 2009). The interruption of *uspA* gene Tn*6018*-MARR-Tn*6018* is considered as the basic structure of AbaR islands, however, the MARR region in different AbaR islands varies considerably in terms of both size and type of cargo genes. Nonetheless, a common feature of all AbaR associated MARR is the presence of a wide variety of resistant determinants including genes associated with mobility, genes for antibiotics, antiseptics, heavy metal resistance genes and efflux associated genes (Kochar *et al.* 2012).

The second common transposon that forms the backbone of AbaR islands is Tn*6021*. The Tn*6021* transposon also has a cluster of five core transposition genes towards its 5' end and three genes *uspA*, *sup* and orf4 towards it 3' end. The *uspA* in Tn*6021* is also a target for Tn*6018*-MARR-Tn*6018*. The third transposon that is also commonly found to form the backbone of AbaR islands is Tn*6022*, which is essentially a Tn*6021* transposon where *sup* gene is interrupted by IS*AbaR1* flanked, bla_{Oxa23} carrying transposon Tn*2006* (Figure 1.1).

As described in above sections, the AbaR islands in various MDR strains of *A. baumannii* differ from each other in terms of length, gene content, and gene sequence due to various events of DNA integration, deletion and rearrangements (Post and Hall 2009). Single or multiple intramolecular recombination events between the identical copies of sequences have been reported in various AbaR islands (Post *et al.* 2010). Various events of gene deletion, gene replacement and gene acquisition mediated by the homologous recombination between copies of IS*26*, copies of Tn*6022* and copies

of *CR2* segments are described as the heart of diversification of AbaR islands (Krizova *et al.* 2011, Harmer *et al.* 2014).

Although the above described three transposons are commonly found in AbaR islands, other complex transposons like Tn*6166*, Tn*6167* and Tn*6168* are frequently being reported to constitute the backbone of AbaR islands (Nigro *et al.* 2011, Nigro and Hall 2012b, Saule *et al.* 2013). Various transposons forming the backbone of AbaR islands and their phylogenetic relationship will be analysed and discussed in chapter 3.

1.2.4 AbaR and Tn*7* transposon similarity

The cut-and-paste bacterial transposon Tn*7* is well studied in many bacterial species (Parks and Peters 2009, K. Y. Choi *et al.* 2013) and its mechanism of target site selection and transposition is well established. Like Tn*7* transposon, the AbaR islands are bounded by 19-26 bp imperfect IR sequences and their integration results in DR sequence at the target. The integration of AbaR islands at *comM* disrupts this gene and forms ACCGC as a DR sequence at the integration site (Adams *et al.* 2010). Towards their 5' end, the AbaR islands also carry a cluster of five genes, *tniA*, *tniB*, *tniC*, *tniD*, *tniE*, that are predicted to be "transposition genes" (Post *et al.* 2010). Bioinformatics analysis showed a striking similarity between the five transposition genes of Tn*7* with the core transposition genes of AbaR1 (Rose 2010). The comparison of nucleotide and protein sequence of these five transposition genes from AbaR1 revealed a distant relation of AbaR islands with a promiscuous transposon Tn*7* (Rose 2010). Therefore, Kochar *et al.* (2012) named the AbaR island in MDR *A. baumannii* strain A424 as Tn*AbaR23* where Tn was used to indicate the relationship with Tn*7* transposon.

1.3 Costs associated with development of resistance and virulence phenotypes in bacteria

It is generally considered that the acquisition or development of antimicrobial resistance is physiologically and energetically costly for the cell. However, studies have

shown that the cost associated with the acquisition of resistance can be, over time, mitigated during growth or by means of other beneficial associations stress free environment and association with host genome (Starikova *et al.* 2013). Enhanced antimicrobial resistance has been found to reduce the growth rate in various bacteria including *E. coli* and *Salmonella* sp. (Smani *et al.* 2012). A study done in a clinical isolate of *A. baumannii* showed that despite no growth cost, the enhanced fluoroquinolone resistance due to overexpression of efflux genes resulted in decreased virulence of the bacteria (Smani *et al.* 2012). A bigger cost of acquiring colistin resistance was observed in *A. baumannii* strain 19606 where the colistin resistant mutants displayed reduced growth rate, reduced *in vitro/in vivo* fitness and attenuated virulence as compared to their parent wild type (Beceiro *et al.* 2014). It is important to note that, development of resistance can be cost free and various factors like epistasis, environmental conditions and compensatory mutations can affect the cost associated with it (Andersson and Hughes 2010).

A majority of AbaR islands described so far in MDR *A. baumannii* strains carry genes and determinants for antimicrobial resistance and genome mobilization. The antimicrobial resistance phenotype associated with these AbaR islands is largely predicted based on their genetic composition. A limited number of experimental studies have reported the role of AbaR islands in the development of drug resistance phenotype. The deletion of AbaR-like island in *A. baumannii* strain A424 was assumed to be associated with enhanced resistance towards ciprofloxacin (Kochar *et al.* 2012) while other reports show no contribution of AbaR islands towards the overall resistance phenotype of the host strain (Krizova *et al.* 2011). Recently, there has been a report on the development of antimicrobial resistance in a susceptible strain when AbaR was conjugally delivered into its genome (Hamidian *et al.* 2014a). In this study, I will be investigating the AbaR-like island in strain A424 aiming to assess its contribution on the phenotypes of the host strain.

1.4 *A. baumannii* strain A424 and its Tn*AbaR23* resistance island

A424 is a multi-drug resistant non-epidemic clinical *A. baumannii* strain belonging to the global clone complex GC I, isolated from a human wound infection in Croatia (Kochar *et al.* 2012). The strain A424 and although the strain is not epidemic, it is clinically important because the clinical isolates from skin or wound infection are considered as the third common isolates in hospital settings after isolates from respiratory tract and blood stream infections (Viehman *et al.* 2014). The strain A424 is fully sequenced and the sequence is deposited in the Sequence Read Archive under accession number SRA045827.2 (Kochar *et al.* 2012).

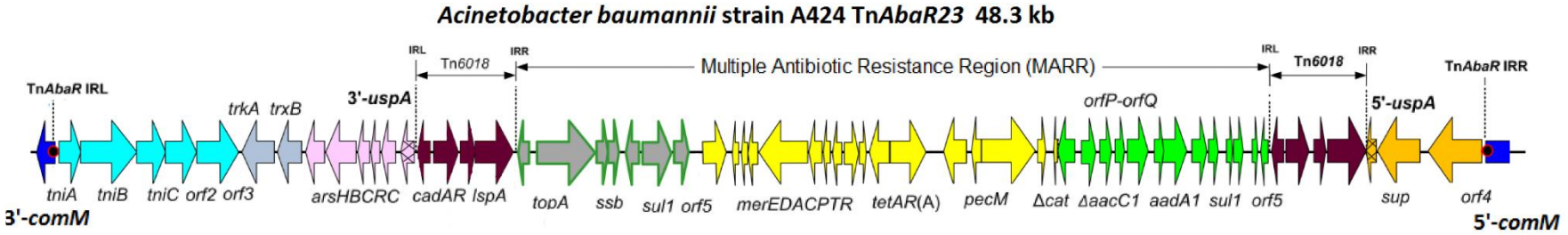

Figure 1.2 Structure of Tn*AbaR23* in *A. baumannii* strain A424.

Tn*AbaR23* possesses Tn*6019* transposon backbone genes *tniABC*, orf2, orf3, putative monooxygenase *trkA*, Thioredoxin reductase *trxB*, arsenic resistance operon genes *arsHBCRC*, sulfate permease gene *sup* and hypothetical gene orf4. The multiple antibiotic region MARR, bracketed by two directly oriented copies of Tn*6018*, is integrated at the universal stress protein gene *uspA*. Adapted from Kochar *et al* (2012).

AbaR island called Tn*AbaR23* is inserted at the chromosomal *comM* gene (Figure 1.2). The strain is found resistant to various antibiotics like tetracycline, carbenicillin, ampicillin, trimethoprim, sulfamethoxazole, and chloramphenicol (Kochar *et al.* 2012). Besides possessing various efflux-associated genes, Tn*AbaR23* island possess a cluster of genes predicted to confer resistance to a wide range of antimicrobial agents.

In 2012, contribution of Tn*AbaR23* to the antibiotic resistance phenotype of host strain A424 was attempted to examine experimentally for the first time (Kochar *et al.* 2012). The 48.3 kb Tn*AbaR23* was deleted *en bloc* and the antibiotic resistance phenotype of the Tn*AbaR23* deleted mutants DCO174 and DCO163 were compared with the parent wild type. Surprisingly and unexpectedly, DCO163 indicated a possible genetic deletion or re-arrangement elsewhere in the genome in a pulsed field gel electrophoresis (PFGE) profile. The second mutant DCO174 on the other hand displayed unusual elevated resistance towards ciprofloxacin. The authors hinted that the mutant DCO174 might have an additional cryptic mutation in the efflux genes that would have resulted in the unexpected ciprofloxacin resistance. Since the Tn*AbaR23* deleted mutant created by Kochar *et al.* (2012) displayed additional mutation/s elsewhere in their genome, the antimicrobial resistance phenotype reported in Tn*AbaR23* deleted mutants by the authors can be considered inconclusive. In the light of additional mutations in both of the mutants DCO163 and DCO174, the antibiotic resistance phenotype reported by the authors cannot be attributed to the carriage of Tn*AbaR23*.
The first attempt to examine the contribution of Tn*AbaR23* on the antibiotic resistance phenotype of the strain A424 can be considered as inconclusive since the authors were unsuccessful in creating a true Tn*AbaR23* deleted mutants for antibiotic resistance phenotype comparison with the parent wild type.

The main aim of this thesis will be to explore and understand the role of Tn*AbaR23* on the phenotype of the host strain A424. Alongside, I will be investigating the features of various AbaR islands reported in *A. baumannii* strains aiming to understand the diversity of AbaR islands. I will be presenting the data on the survey of various AbaR islands in *A. baumannii* genome and attempt to establish a phylogenetic relationship between them. As described in earlier sections, deletion, integration, rearrangement

and gene shuffling are commonly observed phenomenon within the internal region of AbaR elements. In my subsequent result chapter, I will be investigating the plasticity of Tn*AbaR23*. The final result chapter is central to this research. In this chapter, I will be providing data to show how Tn*AbaR23* affects various phenotypes of the host A424. I will be deleting the Tn*AbaR23 en bloc* as described in Kochar *et al.* (2012) aiming to create mutants that do not harbour unintended mutations. I will then use the mutants in various *in vitro* and *in vivo* assays to compare their phenotypes with parent A424 wild type.

2 Aims of the study

The key question I will be addressing is how Tn*AbaR23* contributes on the resistance, fitness and virulence phenotypes of the host strain A424. I will be carrying out a range of experiments to answer this question. Also, I will be surveying various AbaR islands reported in *A. baumannii* and attempt to establish a phylogenetic relationship between them. I will also be examining the stability of the internal region of Tn*AbaR23* in A424 strain

- ➔ Survey of AbaR islands to establish the phylogenetic relationship between the common AbaR islands and novel AbaR-like islands
- ➔ Investigate the plasticity of Tn*AbaR23* and study the contribution of Tn*6018*-associated transposase gene *tnpA* in the stability of internal region of Tn*AbaR23*
- ➔ Construction of *A. baumannii* mutants lacking Tn*AbaR23* transposon
- ➔ Compare the antibiotic resistance phenotype of mutant and parent wild type
- ➔ Compare the fitness phenotype in *in vitro* head-to-head competition, growth and biofilm assays
- ➔ Compare the virulence phenotype using *Galleria mellonella* as an infection model

3 Materials and methods

3.1 Media, reagents and solutions

Brain Heart Infusion (BHI) broth plus 30 % glycerol

USE: Storage of bacterial stocks at -20 and -80°C

47 g of brain heart infusion broth powder (Oxoid) was dissolved in a final volume of 1 l of distilled water containing 30 % (v/v) glycerol, autoclaved before use.

Lysogeny Broth (LB) and Agar (LA)

USE: Standard liquid (LB) and solid growth (LA) medium for bacterial cultures

Lysogeny Broth (LB) was prepared by dissolving 4 g of tryptone, 2 g of yeast extract and 2 g of NaCl in distilled water to a final volume of 400 ml. LB agar (LA) was prepared as for LB with 1.5 % (w/v) of agar, both media types autoclaved before use.

Simmon's Citrate Agar (SCA)

USE: Selection for *A. baumannii* during conjugation experiments

9.2 g of Simmon's citrate agar powder (Oxoid) was dissolved in a final volume of 400 ml distilled water, media autoclaved before use.

Super Optimal broth with Catabolite repression (SOC)

USE: Broth for non-selective outgrowth of bacteria post-transformation

SOC was prepared by dissolving 5 g of tryptone, 2.5 g of yeast extract and 5 g of NaCl into 200 ml of distilled water. After autoclaving, 50 µl of 2 M $MgCl_2$ (filter sterilized) and 200 µl of 1M glucose (filter sterilized) were added to 1 ml of medium.

Tris-EDTA (TE) buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA, pH 8.0

Tris-acetate-EDTA (TAE) buffer

2 M Tris-HCl

2 M Acetic acid

50 mM EDTA

1 M $MgSO_4$

24.6 g $MgSO_4 \cdot 7H_2O$

Distilled water to 100 ml

0.9% NaCl (saline)

9 g NaCl (154 mM final; 0.9% w/v)

Distilled water to 1 litre

Filter sterilise

3.2 Growth conditions for bacteria

Bacterial strains were routinely grown at 37°C on Lysogeny Agar (LA) or Lysogeny Broth (LB) unless otherwise stated. Routine growth in liquid culture was carried out as overnight culture in 2 ml of LB at 37°C and 200 rpm for 16 - 18 h in a 20 ml universal tube unless otherwise stated. The frozen stock of bacteria was maintained at - 20°C and - 80°C on brain heart infusion broth supplemented with 30 % glycerol.

3.3 Strains and plasmids used

A collection of *Acinetobacter baumannii* clinical isolates were thankfully received from Dr Kevin Towner collected at the Queen's medical Centre, Nottingham. Various wild type and mutant strains along with *E. coli* strains and plasmids used are listed in Table 3.1.

Table 3.1 Bacterial strains and plasmid used and created in this study

Bacterial strain/plasmid	**Alternative designation**	**KR designation***	**Description, genotype, characteristic features**	**Reference or source**
A424 WT	A424	KR279	Isolated from wound infection in Croatia	Received from Dr. Kevin Towner
A92	A92	KR109	MDR strain isolated from Spain	Received from Dr. Kevin Towner
S17.1λpir::pJTAG		KR1410	A pJTAG donor strain	Kochar *et al.* 2012
pJTOOL_Apra_*adeS*^{G178A}	pJW-1	KR4202, pKR742	The *adeS*^{G178A} alongside ~750 bp flanking region cloned at the *NotI* and *XbaI* site of pJTOOL-3-Apra (KR3646)	Sapkota N and Wanford J unpublished data 2015
pJTOOL-3-Apra		KR3646	*E. coli* cc118λpir carrying pJTOOL derived plasmid pJTOOL-3-Apra where *cat* gene has been replaced by *aac(3)-IV*-apra gene using lambda red recombination method	David Ngmenterebo unpublished data 2015
A424_pJTAG_R	SCO5	KR3178	Right crossover mutant with pJTAG incorporated in DF of Tn*AbaR23* transposon	This study
A424_pJTAG_L	SCO1	KR3205	Left crossover mutant with pJTAG incorporated in UF of Tn*AbaR23* transposon	This study
A424ΔTn*AbaR23*::*aacC1*_14	DCOA14	KR3075	KR3205 derived double cross over mutant, Tn*AbaR23* transposon swapped with *aacC1* cassette during homologous recombination	This study
A424ΔTn*AbaR23*::*aacC1*_C5-2	DCOC5-2	KR3223	KR3205 derived double cross over mutant, Tn*AbaR23* transposon swapped with *aacC1* cassette during homologous	This study

Bacterial strain/plasmid	Alternative designation	KR designation*	Description, genotype, characteristic features	Reference or source
			recombination	
A424ΔTn*AbaR23*::*aacC1*_C5-4	DCOC5-4	KR3220	KR3178 derived double cross over mutant, Tn*AbaR23* transposon swapped with *aacC1* cassette during homologous recombination	This study
A424ΔTn*AbaR23*::*aacC1*_1-3	DCOA1-3	KR3204	KR3178 derived double cross over mutant, Tn*AbaR23* transposon swapped with *aacC1* cassette during homologous recombination	This study
A424ΔTn*AbaR23*::*aacC1*_174	DCO174	KR1543	Tn*AbaR23* island deleted by allelic exchange, carries *aacC1* cassette, harbours $G_{178}A$ at the *adeS*	Crosatti *et al.* unpublished observation 2013
A424ΔTn*AbaR23*Δ*aacC1-7*	DCOA14-Δ7	KR3873	DCOA14 derived marker less mutant, *aacC1* cassette flipped out	This study
A424ΔTn*AbaR23*Δ*aacC1-18*	DCOA14-Δ18	KR3874	DCOA14 derived marker less mutant, *aacC1* flipped out	This study
A424ΔTn*6018*-MARR-3	ΔMARR-3	KR3326	Spontaneous mutant of A424 wild type that has lost its Tn*6018*-MARR region from the Tn*AbaR23* island	This study
A424ΔTn*6018*-MARR-1	ΔMARR-1	KR3324	Spontaneous mutant of A424 wild type that has lost its Tn*6018*-MARR region from the Tn*AbaR23* island	This study
A424ΔTn*6018*-MARR-2	ΔMARR-2	KR3325	Spontaneous mutant of A424 wild type that has lost its Tn*6018*-MARR region from the Tn*AbaR23* island	This study
A424ΔTn*6018*-MARR-4	ΔMARR-4	KR3327	Spontaneous mutant of A424 wild type that has lost its Tn*6018*-MARR region from the Tn*AbaR23* island	This study

Bacterial strain/plasmid	Alternative designation	KR designation*	Description, genotype, characteristic features	Reference or source
pKOBEG_Apra		KR1479	Temperature sensitive plasmid for use in lambda red recombination	Chaveroche *et al.* 2000
*Escherichia coli*_pFLP2		KR137	*E. coli* strain carrying plasmid pFLP2	H. P. Schweizer Colorado State University
pFLP2_*sul1*	pNS4	pKR631	pFLP2 derived plasmid with *bla* gene swapped with *sul1* gene (A424WT) using lambda red method	This study
pJTAG		KR1358	pJTOOL-3 derived suicide vector carrying the upstream and downstream flanking regions of *comM* gene in AbaR1 island from strain AYE, carry *aacC1* cassette that confers gentamicin resistance and *sacB* gene for sucrose counter selection	Kochar *et al.* 2012
pWH1266-Gm		KR3945, pKR710	~8 kb *E. coli - A. baumannii* shuttle vector used for complementation in *A. baumannii*	(Camarena *et al.* 2010)
A424ΔTn*6018*-MARR-3::pWH1266-GM	ΔMARR-3-pWH1266-Gm	KR4278	A424ΔTn*6018*-MARR_3 strain carrying plasmid pWH1266-GM	This study
A424ΔTn*6018*-MARR-3::pWH1266-GM-1	ΔMARR-3-pWH1266-Gm-1	KR4279	A424ΔTn*6018*-MARR_3 strain carrying plasmid pWH1266-GM carrying the fragment 1 within Tn*6018*-MARR at the *BamHI* and *SalI* site	This study
A424ΔTn*6018*-MARR-3::pWH1266-GM-2	ΔMARR-3-pWH1266-Gm-2	KR4280	A424ΔTn*6018*-MARR_3 strain carrying plasmid pWH1266-GM carrying the fragment 2 within Tn*6018*-MARR at the *BamHI* and *SalI* site	This study
A424ΔTn*6018*-MARR-	ΔMARR-3-	KR4281	A424ΔTn*6018*-MARR_3 strain carrying plasmid pWH1266-GM	This study

Bacterial strain/plasmid	Alternative designation	KR designation*	Description, genotype, characteristic features	Reference or source
3::pWH1266-GM-3	pWH1266-Gm-3		carrying the fragment 3 within Tn*6018*-MARR at the *BamHI* and *SalI* site	
A424ΔTn*6018*-MARR-3::pWH1266-GM-4	ΔMARR-3-pWH1266-Gm-4	KR4282	A424ΔTn*6018*-MARR_3 strain carrying plasmid pWH1266-GM carrying the fragment 4 within Tn*6018*-MARR at the *BamHI* and *SalI* site	This study
A424ΔTn*6018*-MARR-3::pWH1266-GM-5	ΔMARR-3-pWH1266-Gm-5	KR4283	A424ΔTn*6018*-MARR_3 strain carrying plasmid pWH1266-GM carrying the fragment 5 within Tn*6018*-MARR at the *BamHI* and *SalI* site	This study
A424ΔTn*6018*-MARR-3::pWH1266-GM-6	ΔMARR-3-pWH1266-Gm-6	KR4284	A424ΔTn*6018*-MARR_3 strain carrying plasmid pWH1266-GM carrying the fragment 6 within Tn*6018*-MARR at the *BamHI* and *SalI* site	This study
A424ΔTn*6018*-MARR-3::pWH1266-GM-7	ΔMARR-3-pWH1266-Gm-7	KR4285	A424ΔTn*6018*-MARR_3 strain carrying plasmid pWH1266-GM carrying the fragment 7 within Tn*6018*-MARR at the *BamHI* and *SalI* site	This study
ΔMARR-3::pJW1	Δ3_SNP_SCO	KR4263	ΔMARR-3 strain carrying pJW1	This study
ΔMARR_*adeS*^{G178A}	Δ3_SNP_DCO	KR4268	ΔMARR-3 derived strain carrying *adeS*^{G178A}	This study
DCOA14-Δ7::pJW1	Δ7_SNP_SCO	KR4262	DCOA14-Δ7 strain carrying pJW1	This study
DCOA14-Δ7_*adeS*^{G178A}	Δ7_SNP_DCO	KR4267	DCOA14-Δ7 derived strain carrying *adeS*^{G178A}	This study
A424::pJW1	A424_SNP_SCO	KR4261	A424 derived strain carrying pJW1	This study
A424:: *adeS*^{G178A}	A424_SNP_DCO	KR4266	A424 derived strain carrying *adeS*^{G178A}	This study

Bacterial strain/plasmid	Alternative designation	KR designation*	Description, genotype, characteristic features	Reference or source
A424-rev-*adeS*^{G178A}	A424_rev	KR4286	A424 carrying *adeS*^{G178A} in pJW-1 reverted to wild type post sucrose counter-selection	This study
DCOA14-Δ7-rev-*adeS*^{G178A}	Δ7_rev	KR4287	DCOA14-Δ7 strain carrying *adeS*^{G178A} in pJW-1 reverted to DCOA14-Δ7 post sucrose counter-selection	This study
ΔMARR-3-rev-*adeS*^{G178A}	Δ3_rev	KR4288	ΔMARR-3 strain carrying *adeS*^{G178A} in pJW-1 reverted to ΔMARR-3 post sucrose counter-selection	This study
pJTOOL-apra-*tnpA*_L	pJNS5	pKR750	a deletion vector used to target the tnpA from left copy of Tn*6018* in Tn*AbaR23*	This study
pJTOOL-apra-*tnpA*_R	pJNS6	pKR751	a deletion vector used to target the tnpA from right copy of Tn*6018* in Tn*AbaR23*	This study
A424Δ*tnpA*_L	A424Δ*tnpA*_L	KR4254	A424 derived mutant with left Tn*6018-tnpA* deleted	This study
A424Δ*tnpA*_R	A424Δ*tnpA*_R	KR4255	A424 derived mutant with right Tn*6018-tnpA* deleted	This study
A424Δ*tnpA*_L_R	A424Δ*tnpA*_L_R	KR4256	A424 derived mutant with left and right Tn*6018-tnpA* deleted	This study

*KR number corresponds to the physical strain catalogue in Dr Kumar Rajakumar's laboratory at the University of Leicester.

3.4 Genomic DNA extraction

Genomic DNA was extracted by lysing the cells in 500 µl of overnight culture containing ~2×10^9 colony forming units (CFU) using the 5Prime ArchivePure DNA Purification Kit (VWR). Plasmid DNA was extracted by lysing the cells in 500 µl of overnight bacterial broth using GenElute Plasmid Miniprep Kit (Sigma-Aldrich). The plasmids that were to be used for downstream cloning were eluted in 50 µl of PCR grade water. The DNA was stored at – 20°C until used.

3.5 DNA quantification

DNA was quantified using Nanodrop 2000 using DNA elution buffer or PCR grade water as blank where appropriate during quantification.

3.6 Restriction enzymes and restriction digestions

Restriction enzymes were purchased from New England Biolabs (www.neb.uk.com), Roche (www.roche.co.uk), Promega (www.promega.co.uk), or ThermoFisher Scientific (http://www.thermofisher.com). The routine volume of 20 - 50 µl digestion mixture was set up for digestion using appropriate buffers at 1 x concentration and when required 1 x bovine serum albumin (BSA) concentration unless otherwise stated. Following incubation at the appropriate time and temperature, restriction digests were inactivated by heat treatment. Digests were cleaned up to wash buffers and enzymes using PCR DNA purification kit Geneflow and pure DNA were eluted in 20 - 50 µl of PCR grade water.

3.7 Ligation reactions

Ligation reactions were carried using 3U of T4 DNA ligase (Promega) and 10 × T4 DNA ligase buffers. The volume of ligation reaction was in general 10 - 20 µl with insert to vector ratio of at least 2:1. Ligation was done either at 22°C for 3 h or at 4°C for ~16 h.

The ligations were heat inactivated at 70°C for 10 min, vacuum dried and washed twice with 1 ml of 70 % gradient grade ethanol. The washed and dried DNA was then suspended in 5 µl of PCR grade water. The cleaned ligation reaction was then stored at 4°C until use.

3.8 DNA sequencing

DNA samples were sent for Sanger sequencing at GATC Biotech (https://www.gatc-biotech.com). The sample of DNA and primers was prepared and sent according to the company instructions.

3.9 PCR primer design and synthesis

Oligonucleotides for PCR were designed using Primer3 (http://insilico.ehu.es/primer3/) and were synthesized by Sigma-Aldrich (http://www.sigmaaldrich.com).

Primers used in the PCR mapping of AbaR-like island in strain A92 is given in Table 3.2.

Table 3.2 Primers used in the PCR mapping of GI_*comM*_A92

Primer name	**Sequence 5'→ 3'**	**Reference**
1F	CTTAATTGCCTCTGGTCAAC	(Shaikh *et al.* 2009)
2F	TCCATTTTACCGCCACTTTC	(Shaikh *et al.* 2009)
NS1*	TCTGGTGAATTGTCAGGAGC	This study
NS2	ATTTGGCTCCCAACCGATCC	This study
NS3	TCGCCTTTCTCAATTTTGG	This study
NS4	TATCCGAGTGTCAGGGTTCCGC	This study
NS5	TACACGTTCTGCCAGTGAGG	This study
NS6	AACGGTTCAGTGGATTACGC	This study
NS7	AGCCGAGATACCTTCTGCAA	This study
NS8	AAGAATTCGTCTCCGCTTCA	This study
NS9	TGCCGGCCTTTAATGTATTC	This study
NS10	TCGGTATAAGCGGGAACTTG	This study
NS11	GATGACTGTGGTCGCAGTGT	This study
NS12	ATGAACTATGCGACGGAACC	This study
NS13	TTAATTTCCGCTTATCCAAAG	This study
AbaR1UF	CCGCCGTCTTCAACTCTTAG	(Kochar *et al.* 2012)
NS14	TTGCAGAAGGTATCTCGGCT	This study

*The NS primers listed in the table were designed using IS116 sequence as template for this study. The primers are archived in physical catalogue in Dr Kumar Rajakumar' s laboratory at the University of Leicester.

3.10 *In silico* PCR

The sequence of primers was used for *in silico* PCR using the freely available web based tool (http://insilico.ehu.es/PCR/). The size of the PCR product determined by *in silico PCR* and the actual size obtained during the mapping of GI_*comM*_A92 are given below in Table 3.3.

Table 3.3 PCR amplicon sizes obtained during the mapping of GI_*comM*_A92

Primer pair	**Size of the PCR amplicon (bp)**	
	***In silico* PCR with IS116 sequence**	**PCR using A92 DNA ***
1F + NS3	1043	1043
2F + NS3	613	613
1F + NS2	4503	4503
2F + NS2	4073	4073
1F + NS1	8567	8567
2F + NS1	8137	8137
NS1 + NS4	384	384
NS4 + NS5	7320	7320
NS5 + NS6	242	242
NS6 + NS8	7005	7005
NS6 + NS9	8237	8237
NS6 + NS7	9622	9622
NS11 + NS14	6656	6656
NS10 + NS11	734	734
NS10 + NS12	5317	5317
NS12 + NS13	714	714
AbaR1UF + NS13	2100	1484

* The approximate size of the fragments obtained after PCR was estimated by agarose gel electrophoresis.

All the other primers used for various purposes are listed in Table 3.4.

Table 3.4 Primers used in this study

*Primer ID	Alternative name	Sequence 5'→ 3'	Target, features	Reference
PR3031	NS15	TGATTTGCTGGTTACGGTGA	linear MARR detection within Tn*AbaR23*	This study
PR3032	NS16	AGTTCGCGCTTAGCTGGATA		
PR2520	NS17	CATTTCCATGCCGAGTTGTC	Tn*6018*-MARR circular junction detection	This study
PR2521	NS18	TTCCAATCGGAACCCTTGT		
PR1767	ModK-R	CGTAGCAGCCGTTGATAAAGTTGTC	Tn*6018*-MARR deletion detection	Kochar *et al.* 2021
PR1766	ModC-F	CATCCGCGATCATTTGAATCAGTTC		
PR3037	NS19	CAATAAGCGGCCGCTCCCCTGCTCGCGCAGGCTG	Amplify apramycin gene from pKOBEG_Apra, has *NotI* site	This study
PR3378	NS20	GGTGTTGTATAGGCGTAGGTGTCAGTCAGATCC	To amplify the UF *tnpA* in Tn*6018*	This study
PR3379	NS21	GCTTAGCGGCCGCGCCCGGTCCACCGGCCTGATTGG		This study
PR3380	NS22	GGGTGGTATCAGATTGATGGGGTGAAGG	Primer binds immediately after stop codon of Tn*6018*-associated *tnpA* and amplify the DF region of *tnpA*	This study
PR3381	NS23	GCTTAGCGGCCGCCGGGCTAACACGTCGCGCC		
PR3383	NS24	GCTTAGCGGCCGCCCCCTCTCAAGTAATATCGTGATGATTGC	Primer binds immediately after stop codon of Tn*6018*-associated *tnpA* and amplify the DF region of tnpA	This study
PR3543	NS47	GTCTTCGACTAAGTGAGATAAACCTTC	Detects the mutant *adeS* allele from DCO174	This study
PR3544	NS48	GAAGATTGGACCAGTTTTCATTTTGTAG		
PR3752	NS25, 1F	TATATGGATCCAGTGGCTTTTGGAATGG	MARR cloning fragment 1 from Tn*AbaR23*, *BamHI*	This study
PR3753	NS26, 1R	ATTATGTCGACACGCTCGATCTCCTGTAG	MARR cloning fragment 1 from Tn*AbaR23*, *SalI*	This study
PR3754	NS27, 2F	TATATGGATCCGATGGGCCGCACCAAAGCCGA	MARR cloning fragment 2 from Tn*AbaR23*, *BamHI*	This study
PR3755	NS28, 2R	ATTATGTCGACGCTTGGGCCTCCCGC	MARR cloning fragment 2 from Tn*AbaR23*, *SalI*	This study
PR3756	NS29, 3F	AATTAGCGGCCGCTTAACGCCAACCAAGC	MARR cloning fragment 3 from Tn*AbaR23*, *NotI*	This study
PR3757	NS30, 3R	AATAATCTAGAGGCATACCCTAACTTGAT	MARR cloning fragment 3from Tn*AbaR23*, *XbaI*	This study

*Primer ID	Alternative name	Sequence 5'→ 3'	Target, features	Reference
PR3758	NS31, 4F	TATATGGATCCTCGGCGCAGAGCGA	MARR cloning fragment 4 from Tn*AbaR23*, *BamHI*	This study
PR3759	NS32, 4R	ATTATGTCGACATCGCTTGACTCCGTACAT	MARR cloning fragment 4 from Tn*AbaR23*, *SalI*	This study
PR3760	NS33, 5F	AATTAGCGGCCGCGATGAACTCCTGTGA	MARR cloning fragment 5 from Tn*AbaR23*, *NotI*	This study
PR3761	NS34, 5R	AATAATCTAGATTTTCGGGCCTCGCAT	MARR cloning fragment 5 from Tn*AbaR23*, *XbaI*	This study
PR3762	NS35, 6F	AATTAGCGGCCGCGCGATTACAAGACCTCCG	MARR cloning fragment 6 from Tn*AbaR23*, *NotI*	This study
PR3763	NS36, 6R	AATAATCTAGAATCGGTTTTCTTGTTGCC	MARR cloning fragment 6 from Tn*AbaR23*, *XbaI*	This study
PR3764	NS37, 7F	AATTAGCGGCCGCTTTGCAACAGTGCCAA	MARR cloning fragment 7 from Tn*AbaR23*, *NotI*	This study
PR3765	NS38, 7R	AATAATCTAGAAACCATTATTGGCGC	MARR cloning fragment 7 from Tn*AbaR23*, *XbaI*	This study
PR2514	NS39	CATACTCTTCCTTTTTCAATATTATTGAAGC	To amplify the upstream region of *bla* gene from pFLP2	This study
PR2515	NS40	GACCGAAGCGAGACCTTTTTATAGGTTAATGTCATGA TAATAATGG		
PR2822	NS41	CAATAATATTGAAAAAGGAAGAGTATGAAAGGCGTG ACGTGGAAGTCGCC	*sul1* from Tn*AbaR23* in A. *baumannii* strain A424	This study
PR2823	NS42	GAGTAAACTTGGTCTGACAGCTAGGCATGATCTAACC		
PR2824	NS43	GCAGGAAGCGAGTAATCAGG	Universal primer to amplify downstream of *bla* gene from pFLP2	This study
PR2825	NS44	ACTCTTCCTTTTTCAATATTATTG		
PR2826	NS45	CTGTCAGACCAAGTTTACTC	Universal primer to amplify upstream of *bla* gene from pFLP2	This study
PR2827	NS46	AGTTCGGTGTAGGTCGTTCG		

*The PR numbers in primers corresponds to the physical catalogue in Dr Kumar Rajakumar's laboratory at the University of Leicester.

3.11 Polymerase chain reaction (PCR)

Standard PCR was done using either GoTaq polymerase (Promega) or GoTaq® G2 DNA polymerase (Promega). The PCR reaction components were assembled as a master mixture according to the manufacturer's instruction**Error! Reference source not ound.**. The deoxynucleoside triphosphates (dNTPs) were purchased from Bioline UK and PCR grade water was purchased from Sigma. For cloning and sequencing purposes, high fidelity DNA polymerase like KOD hot start polymerase (Merck milipore) was used. For the standard PCR reaction, the reaction components were assembled in 1.5 ml microcentrifuge tube as a master mixture to ensure that all PCR reactions have same reaction components.

3.12 Colony PCR

Colony PCR method was used to screen a large number of colonies by PCR without having to extract their genomic DNA for use as a template. In brief, a distinct colony from a fresh plate was picked using a sterile tooth pick of a sterile pipette tip and was dissolved in 50 µl of PCR grade water in a sterile 1.5 ml microcentrifuge tube. The colony suspension was then boiled at 100°C for 10 min in a heat block to lyse cells after which the suspension was centrifuged at 12000 × g for 3 min. Finally, 1 µl of the supernatant was used as a template in a standard GoTaq PCR reaction.

3.13 Splicing overlap extension (SOE) PCR

To splice the multiple fragments of DNA for cloning purposes, the SOE PCR protocol described by Choi and Schweizer (Choi and Schweizer 2005) was used. In summary, multiple independent fragments containing 5' end overlapping homologies to the other fragments to be spliced were generated using proof reading DNA polymerase. The PCR generated amplicon were mixed in an equimolar ratio in a single PCR reaction (Table 3.5).

Table 3.5 SOE PCR reaction setup

Reagents in Master mixture (Final concentration)	**Volume (µl) for 1 reaction**
10 × Buffer for KOD Hot Start DNA polymerase (× 1)	5
25 mM $MgSO_4$ (1.5 mM)	3
dNTPs (0.2 mM each)	5
Splicing fragment 1 (50 ng)	as required
Splicing fragment 2 (50 ng)	as required
Splicing fragment 3 (50 ng)	as required
Forward primer (0.3 µM) (added after 14^{th} cycle)	1.5
Reverse primer (0.3 µM) (added after 14^{th} cycle)	1.5
KOD Hot Start DNA polymerse enzyme (1U)	1
Nuclease-Free water	To final volume of 50 µl

The PCR reaction mixture was prepared as master mix to ensure that all the reactions have same proportion of components

The PCR reaction was run in the primary thermocycle without primers. In the initial fourteen cycles, the overlapping regions of the fragments should anneal to each other and form a single spliced fragment. The primers that bind to the 5' and 3' end of the fusion product were then added to amplify the new spliced fragment (Table 3.6).

Table 3.6 SOE PCR thermocycling condition

Steps	**Number of cycles**	**Temperature**	**time**
Polymerase activation	1	95°C	2 min
Primary thermocycle without primers			
Denaturation	14	95°C	20 s
Annealing		2°C below the lowest Tm of overlapping region	30 s
Extension		70°C	10 - 25 s/kb of DNA
Cycling after adding primers			
Initial denaturation	1	95°C	20 s
Denaturation	20 - 40	95°C	20 s
Annealing		2°C below the lowest Tm of primers	30 s
Extension		70°C	10 - 25 s/kb of DNA
Final extension	1	70°C	5 - 10 min
Final hold	1	15°C	∞

The primers binding at the extreme ends of the spliced fragments were added after the initial 14 PCR cycles.

3.14 Agarose gel electrophoresis

DNA was visualized under the ultraviolet light by gel electrophoresis on 0.8 % molecular biology grade agarose prepared in Tris-Acetate-EDTA or TAE buffer supplemented with 10 µg/ml ethidium bromide. Loading dye was added in the DNA sample to the final concentration of 1 × prior to loading the sample on the gel. DNA markers like λ-*Hind*III, Generuler DNA ladder, and 2 - log DNA ladder were run alongside the samples for size estimation.

3.15 Pulse Field Gel Electrophoresis (PFGE)

The PFGE protocol described here is adapted from that described with *Pseudomonas aeruginosa* (MacKenzie *et al.* 2005).

3.15.1 Plug preparation

A 1:100 dilution of the overnight broth of the bacteria was inoculated in to 2 ml of LB which was incubated for further 4 - 5 h. The culture was harvested by centrifuging at 3000 x g for 5 min. The pellet was re-suspended in 2 ml sterile Tris-EDTA or TE buffer. Absorbance of the suspension was measured at OD_{540} nm and was adjusted to 3.0 in a final volume of 1 ml TE. Equal volumes of bacterial suspension and 2 % molten (kept at 56°C) low-melt agarose were gently mixed to make a final volume of 500 µl and this was transferred immediately into the plug cast. The plugs were kept at 4°C for 20 min to solidify then recovered and transferred into 0.5 M EDTA–1 % *N*-lauroyl sarcosine buffer or ES buffer containing Proteinase K to a final concentration of 1 mg/ml. The plugs were digested in ES + Proteinase K buffer for 4 h in a shaking water bath at 55°C then recovered and washed five times in a shaking water bath with 20 ml TE to remove Proteinase K. After this, the plugs were stored in TE at 4°C and discarded after 2 months if unused.

3.15.2 Restriction digestion of plugs

Using a clean scalpel, a 2 - 3 mm wide plug was cut for each strain and was transferred to 1.5 ml microcentrifuge tube for digestion with enzyme. The plugs were washed twice with 200 µL of restriction digestion buffer (without enzyme) prior to incubation with enzyme. Each plug was digested with 10U of the restriction enzyme at recommended temperature for 9 h in 200 µl of restriction digestion mix. After digestion, the plugs were recovered and incubated on ice for 30 min with 1 ml of 0.5 × Tris/borate/EDTA) or TBE buffer to wash off the restriction enzyme. The washed plugs and *Saccharomyces cereviseae* DNA marker were sealed on the tooth of the pulsed field gel electrophoresis comb using 1.2 % pulsed field certified agarose gel prepared in 0.5 × TBE buffer. After assembling the casting tray, ~150 ml of 1.2 % agarose gel was poured in the casting tray with comb containing sealed plugs. Once the gel solidified, the comb was removed and the wells were sealed with molten 1.2 % agarose. The gel containing plugs was then transferred inside the CHEF-DR II tank containing 2 L of 0.5 × TBE buffer at 12°C.

3.15.3 Running condition for PFGE

Run time= 28 h

Final pulse = 100

Initial pulse = 50

Volt/cm = 6

Running temperature = 12°C

After completion of the run, the gel was removed from the tray and was stained on rocking stand in 400 ml nanopure water containing 200 µl of ethidium bromide for 2 h. The bands were visualized under UV light and images taken. Where appropriate, the gel was stained overnight and was de-stained in 400 ml nanopure water prior to visualization.

3.16 Preparation and transformation of chemically competent *E. coli* and *A. baumannii* cells for heat shock transformation

The procedure of preparation and transformation of chemically competent *E. coli* and *A. baumannii* cells described here is adapted from the protocol described in Methods in Enzymology (Hanahan *et al.* 1991).

The overnight bacterial culture was re-inoculated in to 100 ml fresh LB at a ratio of 1:100 and incubated as described above for further 4 - 5 h or until absorbance of the culture at 600 nm reached 0.4 - 0.5. The culture was then removed and was kept on ice for 20 min. The cells were harvested by centrifuging at 3000 × g for 10 min at 4˚C. The remainder of the procedure was carried out while working on ice or in cold room at 4°C. The bacterial pellet was first washed with 50 ml of ice cold $MgCl_2$ (100 mM) then with 50 ml of ice cold $CaCl_2$ (100 mM). During each wash, the culture was incubated on ice for 10 min then centrifuged at 3000 × g for 10 min at 4˚C. The final re-suspension was done in 1 ml of ice cold sterile $CaCl_2$ (100 mM) in 15 % glycerol w/v. The batch of competent cells was dispensed as 50 µl aliquots in pre-chilled 1.5 ml microcentrifuge tubes for immediate use or storage at - 80˚C until used.

For transformation, the competent cells were thawed on ice for 10 min. The plasmid or DNA to be used for transformation was also placed on ice for 10 min. To the 50 µl competent cells, 1 - 10 µl of DNA was added and the mixture was incubated on ice for another 30 min. During heat shock transformation, the tube was directly placed in a heat block at 42˚C for 45 s after which the tube was removed and placed on ice for 2 min for recovery. To the transformation mixture, 950 µl of Super Optimal broth with Catabolite repression or SOC broth at room temperature was added and the cells incubated for 1 h. The transformed cells were then plated on appropriate differential media and were left to grow overnight.

3.17 Preparation and transformation of electro-competent *E. coli* and *A. baumannii* cells

The procedure of preparation and transformation of electro-competent *E. coli* and *A. baumannii* cells described here is adapted from the protocol described by William J Dower (Dower *et al.* 1988).

A 1:100 dilution of the overnight culture of the bacteria was grown in 100 ml LB for 4 - 5 h or until the absorbance at 600nm reached 0.4 - 0.5. The culture was kept on ice for 20 min before harvesting by centrifugation at 3000 × g for 10 min at 4˚C. The supernatant was discarded and the pellet washed with 100 ml of ice cold 10 % glycerol followed by centrifugation. The washing step was performed further 4 times using 50 ml, 25 ml, 10 ml and 5 ml of ice cold 10 % glycerol and the final pellet re-suspended in 1 ml of ice cold 10 % glycerol. The batch of competent cells was dispensed as 50 µl aliquots in pre-chilled microcentrifuge tubes for immediate use and storage at - 80˚C.

For electroporation, 100 µl of competent cells were transferred to a pre-chilled 2 mm gap-width electroporation cuvette (Geneflow, UK) and the cells were electroporated in a Genepulser II system (Biorad, UK) with capacitance at 25 mF, resistance at 200 Ω and voltage at 2.5 kV. Post electroporation, 900 µl of SOC at room temperature was added to the cuvette and the cells were transferred to 1.5 ml microcentrifuge tube for recovery at 37 ˚C and 200 rpm for 1 - 2 h. The recovered cells were then plated on the relevant selective agar media.

3.18 Lambda Red Recombination

The Lambda Red Recombinase System employs three key genes from the phage λ red locus *bet* (β), *exo*, and *gam* (γ) (Chaveroche *et al.* 2000). The Exo protein is a 5’ →3’ exonuclease and degrades the 5’ end of linear DNA molecule generating a single stranded 3’ end of the linear DNA. The Bet protein is a single stranded DNA binding protein and binds to the 3’ end of the linear DNA generated by Exo. This binding

promotes the annealing of linear DNA to the complementary DNA. The Gam protein binds to the host RecBCD exonuclease complex and inhibits its exonuclease activity. This ensures that the linear DNA introduced into the host is protected from the host exonuclease activity and that the Bet and Exo proteins can perform their function to assist the homologous recombination. These three genes are under the control of the arabinose-inducible promoter P_{BAD} (Guzman *et al.* 1995) in the apramycin resistance conferring plasmid pKOBEG_Apra. The plasmid pKOBEG_Apra contains *araC* which is under the control of promoter PC. The product AraC is a regulatory protein which binds to L-arabinose and facilitates the initiation of transcription from the P_{BAD} promoter. Once transcription is initiated, the recombinase genes *bet*, *gam* and *exo* are expressed. The presence of L-arabinose is essential for the transcription of P_{BAD} and the subsequent expression of the Lambda Red Recombinase genes which promote homologous recombination. The expression vector pKOBEG_Apra is a temperature sensitive, low copy number plasmid and is lost above 30°C.

This method was used to swap the gene/gene cassette or DNA in the plasmid with the desirable insert in a homologous recombination employing the Gam, Bet and Exo proteins from the plasmid pKOBEG_Apra. The plasmid pKOBEG_Apra was electroporated into the *E. coli* strain carrying the plasmid of interest to be mutagenized. The electroporated *E. coli* cells were recovered in 950 µl of SOC at 30°C for 1.5 h in an incubator shaking at 200 rpm. After the recovery, the culture was plated on agarose plate containing 30 µg/ml apramycin. The plates were incubated at 30°C for 48 h. The *E. coli* cells were then verified for the presence of two plasmids.

For the induction of lambda red genes in pKOBEG_Apra, the overnight culture of *E. coli* carrying both plasmids was re-inoculated into 100 ml LB containing 30 µg/ml apramycin and the appropriate second antibiotic. The culture was allowed to grow until OD_{600} 0.2 then induced with arabinose at the final concentration of 0.2 % (w/v) to express *gam, bet and exo* genes then the culture was allowed to grow until the absorbance at 600 nm (OD_{600}) reaches 1 after which washed several times with 10 % glycerol as described above to make them electro-competent.

The insert to be cloned was constructed by amplifying 450 - 550 bp regions immediately upstream and downstream of the cloning site in the vector of interest and sowed with an appropriate resistance gene in a SOE PCR. The arabinose induced *E. coli* carrying two plasmids was then electroporated with 20 ng of insert using the Genepulser II system (Biorad, UK) under the conditions described earlier. After electroporation, the cells were recovered in SOC as described above. The recovered cells were plated on LA supplemented with appropriate antibiotic and were incubated overnight. The transformed cells were verified for the presence of insert. The pKOBEG_Apra plasmid is lost on incubation at 37°C.

3.19 Conjugations

For the conjugative delivery of plasmids into *Acinetobacter baumannii* strains, an adapted version of the mating method described by Dennis and Zylstra (Dennis and Zylstra 1998) was used. In brief, single colonies from the donor and the recipient strains were picked from overnight plates and inoculated into 2 ml LB (100 µg/ml gentamicin supplemented for the *E. coli* donor) then grown overnight. A 1:100 dilution of both cultures of donor and recipient were then re-inoculated in 2 ml fresh LB for 6 h after which, the bacteria were harvested by centrifugation at room temperature for 10 min. The pellets were washed by re-suspending in 1 ml/tube pre-warmed LB. Bacteria were washed twice to remove the excess antibiotics. After the second wash, bacterial pellets were re-suspended in 500 µl of pre-warmed LB. The donor and recipient were mixed in 1:1 ratio and the mixture was centrifuged at 3000 x g for 10 min. The pellet was then re-suspended in 30 µl of pre-warmed sterile 10 % glycerol.

The mixed bacterial suspension was then spotted on the centre of pre-warmed LA plate. The spot was allowed to dry and the conjugation plate was incubated overnight. Donor-donor and recipient-recipient conjugation was set up as a conjugation control. The next day, the conjugation spot was collected in 600 µl of sterile 10 % glycerol. Serial dilution of the collected culture was made up to 1:100. The transconjugants were selected on Simmon's citrate agar [SCA] plate supplemented with appropriate

concentration of gentamicin and were verified by PCR for being the expected transconjugant.

3.20 Growth Curves

The overnight broth culture of the strains was diluted 1:100 in broth medium. In 96 well polystyrene flat bottom plates (NUNC, Roskilde, Denmark), a 200 µl aliquot of the prepared suspension was added per well and the plate incubated in the shaking Varioskan® flash spectral scanning multimode reader with continuous shaking at 37°C for 24 h measuring the absorbance at 600 nm every 10 min. The size of the initial inoculum was determined by plating the appropriate dilution of the bacterial suspension on agar media and counting the CFU the following day.

3.21 Bioinformatics tools

Following bioinformatics tools were used in this study

Blastn: To search DNA database for similar DNA sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi)

ApE, A plasmid Editor v2.0.47: To edit DNA sequence in Genbank format files, virtual digests and sequence annotation (http://biologylabs.utah.edu/jorgensen/wayned/ape/)

Finch TV: To view sequence trace file (http://finchtv.software.informer.com/1.4/)

ClustalW: To visualize multiple sequence (nucleotide and protein) alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/)

***In silico* PCR**: Online program to perform *in silico* PCR (http://insilico.ehu.es/)

Oligo Calc: for Oligonucleotide analysis (http://simgene.com/OligoCalc)

Graphpad Prism version 6.00 for windows: For the analysis of data and graph construction (www.graphpad.com)

Phylogenetic analysis was done using MEGA version 6 (Tamura *et al.* 2013).

3.22 Plasmids maps and *in silico* construction of mutant genomes

Plasmid maps and genomes were visualized and constructed using Snapgene (www.snapgene.com), pDRAW32 (http://www.acaclone.com) and geneious (www.geneious.com) softwares.

3.23 Biofilm Assay for *A. baumannii*

The biofilm assay for *Acinetobacter* was adapted from Malloy *et al.* (Malloy *et al.* 2005). From the overnight bacterial plate, a single colony was inoculated into a broth media which was further grown overnight. The following day, 1:100 dilution of overnight culture was inoculated into 2 ml LB. The culture was grown to OD_{600} 2.0 then diluted 1:200 in sterile LB. In the wells of flat bottom 96-well polystyrene plate (NUNC, Roskilde, Denmark); 200 µl aliquots of the suspension were dispensed per well with three replicates per strain. In order to control the quality of assay, CFU of the bacterial suspension at OD_{600} 0.2 was determined by plating the appropriate dilution on LA.

The plates were sealed to avoid evaporation and incubated at 37°C. The plates were harvested after 24 h, 48 h and 120 h to estimate the biofilm formed. The medium was discarded and the plates were washed three times by gentle immersion in phosphate buffer saline or PBS. After the third wash, the plates were air dried for 30 min. The plates were then stained using 200 µl per well of 1 % w/v Crystal Violet. The staining plates were left at room temperature for 15 min and were washed with PBS until the wash was clear. The plates were air dried at room temperature for 30 min in an inverted position. The biofilm stain was then dissolved in 95 % ethanol (200 µl per well) and the absorbance of the dissolved stain was measured at OD 595 nm using the enzyme-linked immunosorbent assay or ELISA plate reader. The experiment was repeated independently three times.

3.24 Determination of minimum inhibitory concentration, MIC, by broth microdilution

The protocol for the antimicrobial MIC by broth microdilution described here is in accordance to the Clinical and Laboratory Standards Institute (CLSI 2009). In brief, from an overnight plate, 2 - 3 isolated colonies the bacteria were aseptically suspended in 3 ml of sterile saline (0.9 % NaCl). The turbidity of the suspension was then matched to a McFarland standard 0.5 (~1 - 2 x 10^8 CFU/ml).The McFarland adjusted suspension was diluted 1:100 in saline and added in 100 µl aliquots to a 96 well flat bottom NUNC plate (with lid) together with antibiotic at varying concentrations. The plate was sealed and incubated at 37°C overnight. At least three replicate dilutions were examined for each strain tested. The purity of the inoculum was also confirmed. The 96 well plates were examined the following day and each well carefully checked for turbidity to determine the MIC of the antibiotic used in the test.

3.25 Antibiotic susceptibility by Etest and disc diffusion method

From the overnight plate, 2 - 4 colonies were picked and were suspended in sterile 0.9 % NaCl. The turbidity of the suspension was adjusted to match 0.5 McFarland standards and used within 15 min of preparation. After a further 1:100 dilution in sterile 0.9 % NaCl the suspension was streaked on iso-sensitest agar to create a semi-confluent growth using a sterile cotton swab. The inoculum was allowed to dry for 10 min before applying the antibiotic discs or Etest strips. The discs and Etest strips were then applied taking care to avoid bubbles between the agar and disc and the plates incubated at 37°C for 20 h. The plates with uneven growth were discarded. The zone of growth inhibition around the discs was measured using a ruler. For Etest, the MIC (µg/ml) was read from the scale on the Etest strip where the symmetrical inhibition ellipse edge intercepts the strip. BSAC version 2013 guideline (Wootton 2013) was referred to interpret the MIC and zone diameter breakpoints for *Acinetobacter*.

Antibiotic abbreviation and the concentration is shown in parenthesis: Tetracycline [TET (10 µg/ml)], Imipenem [IMP (10 µg/ml)], Carbenicillin [CAR (100 µg/ml)], Ampicillin [AMP (25 µg/ml)], Cefotaxime [CTX (30 µg/ml)], Streptomycin [STR (10 µg/ml)], Tobramycin [TOB(10 µg/ml)], Trimethoprim [TMP (2.5 µg/ml)], Chloramphenicol [CHL (10 µg/ml)], Rifampicin [RIF (2 µg/ml)], Sulfamethoxazole/Trimethoprim [SXT (25 µg/ml)], Ciprofloxacin [CIP (5 µg/ml)], Amikacin [AMK (30 µg/ml)], Gentamicin [GEN (10 µg/ml)], Sulfamethoxazole [SUL (25 µg/ml)].

3.26 Sucrose Counter-Selection Method

A single well-grown colony of the bacteria containing counter-selectable vector (integrated into the genome or existing as a free plasmid) was aseptically inoculated in 2 ml LB to grow overnight. The following day, a 1:100 dilution of overnight culture was re-inoculated into fresh 2 ml LB which was further incubated for 4 h. The culture was harvested and then diluted to OD_{600} 0.2 in 1 ml volume. This suspension was serially diluted up to 10^{-5} and the 10^{-1} and 10^{-2}dilutions plated on LA supplemented with 6 % sucrose. The lower dilutions (10^{-4} and 10^{-5}) were plated on LA to estimate the CFU count of the undiluted cell suspension. Plates were then incubated overnight. The colonies growing on sucrose were tested for loss of the *sacB* gene carrying vector from their genome.

3.27 Head-to-head *in vitro* competition between wild type and mutants

The protocol for *in vitro* head-to-head competition assay was adapted from Fernandez *et al.* (Fernandez *et al.* 2012). In short, frozen stocks of the competing strain were recovered and streaked twice before commencing the experiment. A single, fully grown colony was picked and inoculated in 2 ml LB to grow overnight. 1:100 dilution of the overnight culture was re-inoculated in 2 ml LB and was left to grow for 4-6 h. The culture was harvested then re-suspended in 2 ml of 10 mM $MgSO_4$ to adjusted the OD_{600} 0.2. The suspensions of competing strains at OD_{600} 0.2 were then serially diluted

up to 10^{-5}. A 100 µl aliquot of competing strains at 10^{-2} dilution were mixed and then inoculated into 2 ml LB in a 20 ml plastic universal tube. The competition culture was left to grow overnight and 1:100 dilution was sub-cultured in 2 ml LB the following day. Control cultures were maintained by growing the competing strains alone in broth under identical growth conditions with the same sub-culturing protocol. The cultures were sampled at the specified time points to determine the population of competing strains by counting CFU on LA and LA supplemented with the appropriate antibiotic.

3.28 *Galleria mellonella* killing assay

The *G. mellonella* killing assay described here is modified from Peleg (2009).

Inoculum preparation

A suspension of bacteria at OD_{600} 0.2 was prepared following the protocol as per described in head-to-head *in vitro* competition assay. Appropriate dilutions of the OD adjusted suspension were prepared as an inoculum and kept on ice until required. The bacterial load in the inoculum was determined by CFU count.

***Galleria* preparation**

Galleria larvae at the sixth instar stage were used for the experiment. Larvae were ordered from LiveFood UK Limited (LiveFood) and kept in the dark at room temperature and were used within 2 weeks of the shipment. On the day of inoculation, larvae weighing ~250 mg were recovered and grouped (15 per group) in sterile petri dishes. These groups were held at room temperature in dark for 2 h before inoculation.

***Galleria* inoculation**

Individual larvae were held head down in the 96-well flat bottom NUNC plates during the process of inoculation. The rear left pro-leg of the larva was disinfected using a cotton swab dipped in 70 % ethanol. Using a BD micro-fine 1 ml syringe affixed on a repetitive pipette from Stepper™ series, 20 µl of the inoculum (containing roughly 10^4

bacteria) was injected into the haemocoel via the rear left pro-leg. After injection, larvae were incubated at 37°C, in darkness. Two control groups of larvae were maintained; first group contained larvae inoculated with 20 µl of 10 mM $MgSO_4$ and second group included untouched larvae.

Observation

Mortality of the larvae was recorded over a period of 5 days. They were considered dead if no movement was elicited upon touch. The experiment was discarded if there were 2 deaths in any of the control groups (untouched and 10 mM $MgSO_4$ injected group).

3.29 Estimating the proportion of A424 cells with spontaneous deletion within Tn*AbaR23*

A semi-quantitative PCR based method was used to estimate the proportion of cells where the deletion and excision of region within the chromosome of strain A424 had occurred.

A stock solution of 2 µg of A424 gDNA per 5 µL of PCR grade water was prepared. A series of 1/10 dilutions of the gDNA stock solution was set up to obtain dilutions with 200 ng/5 µL, 20 ng/5 µL , 2 ng/5 µL, 0.2 ng/5 µL, 0.02 ng/5 µL, and 2 pg/5 µL water. PCR was set up using 5 µl of the gDNA template at various dilutions in each reaction. Detailed calculation is given in the relevant result section. The detection of PCR amplicon in diluted gDNA was used to determine the proportion of cells where deletion or excision of Tn*6018*-MARR had occurred.

3.30 AbaR-islands survey

To perform the survey of AbaR islands, the nucleotide and amino acid sequences of Tn*AbaR23*-associated genes were used to query NCBI database (Table 3.7) were also used as query sequence in the survey. Both putative and characterized AbaR islands

were located in the genome of various *A. baumannii* strains and their boundaries were delineated. Following parameters were used for blastn and blastp searches.

Table 3.7 Query sequences for AbaR survey.

Gene/region within Tn*AbaR23*	**Tn*AbaR23* loci**	**Length of sequence used**
tniA	3056..3766	710 bp
tniB	3767..5677	1910 bp
tniC	5682..6602	920 bp
tniD	6632..7747	1115 bp
tniE	7725..9188	1463 bp
IRL Tn*AbaR23* to *tniE*	2901..9188	6287 bp
sup to IRR Tn*AbaR23*	47568..51246	3678 bp

Blastn parameters

The query sequence was analysed by blastn with following parameters:

Database: The nucleotide collection (nr/nt) database was selected for search. This database includes Genbank, EMBL, DDBJ, PDB, RefSeq sequences but excludes EST, STS, GSS, WGS, TSA, patent sequences as well as phase 0, 1, and 2 HTGS sequences.

Organism: The database was limited to search within *A. baumannii* (taxid: 470)

Blastn algorithm: Following algorithm parameters were set for blastn: max target sequences 100, short queries tick automatically adjust parameters for short input sequences, expect threshold 10, word size 28, max matches in a query range 0, scoring parameters match/mismatch scores 1-2, gap costs linear, filter tick the low complexity regions, mask tick mask for lookup table only

Blastp parameters

The amino acid sequence of TniA, TniB, TniC, TniD, TniE, Orf4 and Sup from Tn*AbaR23* and the sequence of concatenated proteins TniABCDE and Sup-Orf4 were used as a query sequence in the blastp analysis. Following algorithm and parameters were set for the analysis.

Database: All non-redundant Genbank CDS translations, PDB, SwissProt, PIR, PRF excluding environmental samples from WGS projects.

Blastp algorithm: maximum number of aligned sequences to display 100, automatically adjust word size and other parameters, expect threshold 10, word size 3, maximum matches in a query range 0, scoring matrix BLOSUM62, gap costs existence 11 extension 1 and conditional compositional score matrix adjustment.

3.31 Statistical Analysis

The statistical analysis in growth curve and biofilm assays was performed by either one-way-ANOVA (for comparison of more than two groups) and by Two-way-ANOVA (for comparison of two groups). The survival curve in the *Galleria* killing assay was analyzed by Mantel-cox test. The p values less than 0.05 were considered as statistically significant. In *in vitro* head-to-head competition, at each time point (T), the percentage of competing strains was calculated by counting CFU of the competing strains on selective and non-selective plates. The competitive index [CI] at any time point T was then calculated by dividing the ratio of A424 wild type by the ratio of mutant bacteria in the culture.

$$\mathrm{CI} = \frac{\text{Ratio of wild type at time T}}{\text{Ratio of mutant at time T}}$$

4 Survey of AbaR, the *A. baumannii* resistance island

4.1 Background

Recent advancement in the field of DNA sequencing has facilitated a rapid and comprehensive study of several *A. baumannii* genomes that led to the identification of several AbaR islands in these bacteria. As described earlier in introduction section, the complex structure of AbaR island is built on the backbone transposons like Tn*6019*, Tn*6021* and Tn*6022*. The Tn*6019* backbone is found in AbaR3-like islands whereas Tn*6021* and Tn*6022* backbone is found in AbaR4-like islands. In recent years, complex and divergent AbaR islands are being reported in MDR *A. baumannii* strains that appear novel and distinctive from commonly known AbaR3 or AbaR4 like islands.

Shaikh *et al.* (2009) reported the presence of *comM* associated AbaR-like DNA sequence in *A. baumannii* strains A92 and A25 that appeared distinct from known AbaR-like islands. The sequence of *comM*-associated DNA in strain A25 shared only 88% identity with the terminal sequence of AbaR1 island whereas the sequence of *comM*-associated DNA in strain A92 showed "no match" with the genome database at nucleotide and protein level. Therefore, the authors hypothesized that the *comM*-associated divergent DNA in strains A25 and A92 could be two distinct lineages of AbaR family. Based on the basic architectural difference between AbaR3 and AbaR4 islands, Hamidian and Hall (2011) also proposed AbaR3 and AbaR4 as two different lineages of AbaR islands. Recently identified island AbaR27 in MDR *A. baumannii* strain BJABO715 was also reported as a distinct AbaR4-like island that lack the key AbaR4 genes *uspA* and *sup* (Zhu *et al.* 2013).

Despite the ongoing debate on lineages of AbaR-like islands, a phylogenetic study that encompasses all the known diverse AbaR islands is lacking. This chapter sets out with a specific aim to establish a phylogenetic relationship between AbaR-islands known to-date. The internal architecture of AbaR islands is very diverse, but as described previously, they are built on the backbone of other transposons. In this study, I will be analysing the backbone transposons genes of AbaR-like islands reported in various *A. baumannii* strains to study their relatedness. I will then select transposon backbones for phylogenetic studies ensuring an unbiased representation of all known AbaR

islands. I will subsequently establish a phylogenetic relationship between the representative AbaR-backbone transposons and, by extension, AbaR islands based on the sequence similarity of their transposition genes *tniA, tniB, tniC, tniD* and *tniE*.

4.2 Aims and objectives

The published literatures and Genbank sequence will be used as a source of data for this survey and various bioinformatics tools will be used for the nucleotide and amino acid sequence analysis. In order to establish a phylogenetic relationship between AbaR islands, I will be carrying out following set of experiments and exercise

- ➔ Search the published literature and compile known AbaR-like islands in *A. baumannii*
- ➔ Search the Genbank database to identify putative AbaR-like islands in *A. baumannii* using the nucleotide and amino acid sequences of *tniA, tniB, tniC, tniD, tniE, sup* and orf4 genes from Tn*AbaR23* island as a query sequence
- ➔ Search published literatures and Genbank sequences to identify the transposon that constitutes backbone of AbaR islands compiled during the survey
- ➔ Compare the sequences of transposition genes in the representative backbone transposons to build a phylogenetic relationship

4.3 Result

4.3.1 Survey of AbaR-like islands in *A. baumannii*

The Genbank database and published literatures were searched from August 2014 to April 2015 to identify the AbaR islands reported in *A. baumannii*. The AbaR-like islands reported after this period, are not included in this study.

A total of 58 AbaR-like islands that were unique to each other (in terms of their integration site, genetic composition and host strain) were found represented in 56 *A. baumannii* strains (Table 4.1). Rose (2010) has reported the presence two AbaR

islands, both at non-*comM* site, in the strain A473 but those AbaR islands are not included in this study due to the unavailability of sequence data. Three strains A85 GC1, AB0057 and BJABO715 were also reported to carry the AbaR island integrated at a non-*comM* loci. A novel AbaR island in strain MDR-TJ has been identified in this study that has not integrated within the *comM* gene of the strain. A MDR strains AB0057 was reported to carry AbaR3 and AbaR4 on its chromosome while another MDR strain A85 clone GC1 strain was found to possess a copy of AbaR3 on its chromosome and a copy of AbaR4 on its plasmid pA85-3. The smallest AbaR island was reported in A9380 (9.1 kb) while AbaR26 in strain BJABO868 was the largest island identified so far (120 kb). During this survey, previously unreported putative AbaR-like islands were identified in 7 different *A. baumannii* strains. The boundaries of these putative AbaR-like islands were delineated based (whenever applicable) on the presence of AbaR terminal inverted repeat (IR) sequences and the putative AbaR islands were given interim name GI _strain-name. Where applicable, the backbone transposons of the putative AbaR-islands were also identified based on the sequence comparison with other AbaR backbones. The *comM* integrated resistance islands in strains A92 and A25 reported by Shaikh *et al.* (2009) were also assigned interim names as GI_*comM*_A92 and GI_*comM*_A25 respectively.

Table 4.1 Distribution of AbaR islands in the *A. baumannii* strains

Element	**Accession no.**	**Size in kb**	**Strain**	**Backbone transposon**	**References**
AbaR0	KF483599.2	65	WM98	Tn*6019*	(Hamidian *et al.* 2014b)
AbaR0	CP000521	13	ATCC17978	Tn*6021*	(Smith *et al.* 2007)
AbaR1	CU459141.1	86.2	AYE	Tn*6019*	(Fournier *et al.* 2006)
AbaR2	CP000863.1	19	ACICU	Tn*6019*	(Iacono *et al.* 2008)
AbaR3	KC118540.6	64.4	A85	Tn*6019*	(Kenyon and Hall 2013)
AbaR3	CP001182.1	63	AB0057	Tn*6019*	(Adams *et al.* 2008)
AbaR4	JN107991.2	16.8	D36	Tn*6022*	(Hamidian and Hall 2011)
AbaR4[#]	NC_011586	16	AB0057	Tn*6022*	(Adams *et al.* 2008)
AbaR4-type	HQ700358.1	13.9	AB210	Tn*6022*	(Hornsey *et al.* 2011)
AbaR4[#]	KJ493819.1	~15	A85_pA85-3 clone GC1	Tn*6022*	(Hamidian *et al.* 2014a)
Tn*AbaR4*a	JN129845.1	18	LT-3	Tn*6021*	(Seputiene *et al.* 2012)
AbaR4b	JN129846.1	22	LT-11	Tn*6021*	(Seputiene *et al.* 2012)
AbaR4c	JN129847	20.8	LT-V1	Tn*6021*	(Seputiene *et al.* 2012)
AbaR4d	CP001921	34.6	1656-2	Tn*6021*	(Seputiene *et al.* 2012)
AbaR4e	CP002522	45	TCDC-AB0715	Tn*6021*	(Seputiene *et al.* 2012)
AbaR5	FJ172370.5	56.3	3208	Tn*6019*	(Post and Hall 2009)
AbaR6	GQ406245.5	27.3	D2	Tn*6019*	(Post *et al.* 2010)
AbaR7	GQ406246.3	20	A92	Tn*6019*	(Post *et al.* 2010)
AbaR8	HM590877.5	29	D13 clone GC1	Tn*6019*	(Post *et al.* 2012)
AbaR9	ADGZ00000000	39.4	AB056	Tn*6019*	(Adams *et al.* 2010)
AbaR10	DHA00000000	30.5	AB058	Tn*6019*	(Adams *et al.* 2010)
AbaR11	JF262167.1	19.8	NIPH470	Tn*6019*	(Krizova *et al.* 2011)
AbaR12	JF262168	38	LUH6013	Tn*6019*	(Krizova *et al.* 2011)
AbaR13	JF262169	44.8	6015	Tn*6019*	(Krizova *et al.* 2011)

Element	Accession no.	Size in kb	Strain	Backbone transposon	References
AbaR14	JF262170.1	21.5	LUH5881	Tn*6019*	(Krizova *et al.* 2011)
AbaR15	JF262171.1	54.7	LUH6125	Tn*6019*	(Krizova *et al.* 2011)
AbaR16	JF262172	38.6	LUH7140	Tn*6019*	(Krizova *et al.* 2011)
AbaR17	JF262173	57.5	LUH8592	Tn*6019*	(Krizova *et al.* 2011)
AbaR18	JF262174	51.9	NIPH2713	Tn*6019*	(Krizova *et al.* 2011)
AbaR19	JF262175	30.5	NIPH2554	Tn*6019*	(Krizova *et al.* 2011)
AbaR20	HM357806.1	63	HK302	Tn*6019*	(Krizova and Nemec 2010)
AbaR21	KM921776.1	~64	RUH875 (A297)	Tn*6019*	(Nigro *et al.* 2011)
AbaR22	CP001937.1	38.6	MDR-ZJ06	Tn*6021*	(Zhou *et al.* 2011)
Tn*AbaR23*	JN676148.1	48	A424	Tn*6019*	(Kochar *et al.* 2012)
AbaR23	JN409449.3	52	D81 clone GCI	Tn*6019*	(Kenyon and Hall 2013)
AbaR24	JN968482.3	~54	A1 clone GC1	Tn*6019*	Hamidian unpublished, 2014
AbaR25	CP003846.1	121.7	BJAB07104	Tn*6167* like	(Zhu *et al.* 2013)
Tn*6167*	JN968483.3	37	A91 clone GC2	Tn*6167*	(Nigro and Hall 2012b)
Tn*6166*	JN247441.4	17	RUH134	Tn*6166*	(Nigro and Hall 2012a)
AbaR25	JX481978.1	46.4	K51-65	Tn*6167*	(Saule *et al.* 2013)
ΔAbaR25	JX481979.1	41	K51-74	Tn*6167*	(Saule *et al.* 2013)
AbaR26	KC665626.1	21	D30	Tn*6019*	Hamidian and Hall unpublished, 2013
AbaR26	CP003849.1	~120	BJAB0868	Tn*6167*	(Zhu *et al.* 2013)
AbaR27[#]	CP003847.1	15.3	BJAB0715	NK	Zhu, Yan *et al.* 2013)
GI_*comM*_A25	FJ406499.1	NK	A25	Tn*6166* like	This study, (Shaikh *et al.* 2009)
GI_*comM*_IS116	NZ_AMGF01000002.1	30.6	IS-116	Tn*6267*	This study
GI_*comM*_A92	FJ406500.1	30	A92	Tn*6267*	This study, (Shaikh *et al.* 2009)
GI _A30	CP007577.1	207.9	AC30	Tn*6166*-like	This study, Lean *et al.* unpublished, 2015
GI _AC29[#]	CP007535.2	91.6	AC29	Tn*6166*-like	This study, Lean *et al.* unpublished, 2015
GI _AC12	CP007549.1	NK	AC12	Tn*6166*-like	This study, (Gan *et al.* 2012)

Element	Accession no.	Size in kb	Strain	Backbone transposon	References
Tn*6168*#	JX844630.1	34.4	A9337	Tn*6022*	Chen *et al.* unpublished, 2013
Tn*6022*Δ2	JX844629.1	9.1	A9380	Tn*6022*	Chen *et al.* unpublished, 2013
Reported as being identical to Tn*2006*	AP013357.1	17.6	NCGM 237	Tn*6022*	(Tada *et al.* 2014)
Reported as a "resistant island"	CP003856.1	41.4	TYTH-1	Tn*6166*-like	(Liou *et al.* 2012)
GI_ MDR-TJ#	CP003500.1	26.3	MDR-TJ	Tn*6166*-like	This study, (Gao *et al.* 2011)
GI_ PKABO7	CP006963.1	21.7	PKAB07	Tn*6166*-like	This study, (Saranathan *et al.* 2014)
Tn*6167*	GQ914990.1	37	A94	Tn*6167*	(Nigro and Hall 2012b)
GI_ IOMTU 433	NZ_AP014649.1	16.8	IOMTU 433	Tn*6022*	This study, Tada *et al.* unpublished, 2014

A total of 56 *A. baumannii* strains were reported to carry AbaR-like island and were numbered from AbaR0 to AbaR27 in the published literatures. Some of the AbaR-like islands were simply reported as "resistance island" or were given a transposon number as in the case of strains A94, A9380, A9337, RUH134 and A91 clone GCI. The AbaR islands marked with #represent non-*comM* integration in the genome. The AbaR island in strains A92 and IS116 were given a transposon number Tn*6267* in this study. Strains reported to have AbaR-like island in the published literature with no sequence deposited in Genbank database are excluded in this study. NK = not known due to insufficient information in Genbank database and published literature.

The AbaR-like elements listed in Table 4.1 were interrogated for their constituent backbone transposon as far as possible using either published literatures (where sequence is unavailable) or their sequence comparison. The backbone transposon in AbaR27 is referred here as not known [NK]. Summary of the backbone transposons found in AbaR-like elements are given in Table 4.2. A total of 26 out of 58 (~45%) of the AbaR-islands had transposon Tn*6019* as their backbone. The second most common transposon backbones were found to be Tn*6022* and Tn*6166* which appeared in 8 AbaR elements ~13% of AbaR elements. A total of 7 AbaR elements were found to possess Tn*6021* (12%). The transposon Tn*6167* was found represented in 6 AbaR elements (10%). The unknown backbone transposon constituting AbaR27 was found to be represented in BJABO715 strain only.

Interestingly, the 693 bp DNA sequence of GI_*comM*_A92 deposited in the Genbank also shared 100% identity with the *comM* associated DNA in MDR *A. baumannii* strain IS116 isolated from Iraq. This newly identified island in IS116 was given an interim name GI_*comM*_IS116. However, the 5' end of GI_*comM*_A92 was reported to be harbouring a deletion (Shaikh *et al.* 2009). The strain A92 was physically available in the laboratory and full sequence of IS116 was available in the Genbank database. Before assuming that GI_*comM*_IS116 and GI_*comM*_A92 were identical and shared common transposon backbone, the GI_*comM*_A92 was PCR-mapped using the primers designed on GI_*comM*_IS116 sequence. Primers are listed in material and method section Table 3.2. The backbone of GI_*comM*_IS116 and GI_*comM*_A92 were assigned a transposon number Tn*6267* during this study. In the subsequent sections, Tn*6267* will be used to represent the *comM* associated AbaR island backbone in strain A92 and IS116.The transposon Tn*6267* was so far found as a backbone transposon in the genomic island integrated into *comM* gene in strains A92 and IS116.

Table 4.2 Representation of backbone transposons in various AbaR islands

Transposon backbone	**Number of times represented, type of associated AbaR island**
Tn*6019*	26, AbaR3-like islands
Tn*6022*	8, AbaR4-like islands
Tn*6166*	8, GI_*comM*_A25
Tn*6021*	7, AbaR4-like islands
Tn*6167*	6, AbaR4-like islands
Tn*6267*	2, GI_*comM*_IS116, GI_*comM*_A92
AbaR27	1, AbaR27

During the nucleotide sequence comparison, the 1791 bp sequence of GI_*comM*_A25 deposited in the Genbank deposited share 100% nucleotide and amino-acid sequence identity with transposon Tn*6166* in strain RUH134. Based on this sequence identity with Tn*6166*, the GI_*comM*_A25 was assumed to be built on the backbone of transposon Tn*6166*. Since a full sequence of transposon Tn*6166* was available in the database, Tn*6166* was included in the subsequent phylogenetic study to represent the AbaR island in strain A25 reported by Shaikh *et al.* (2009).

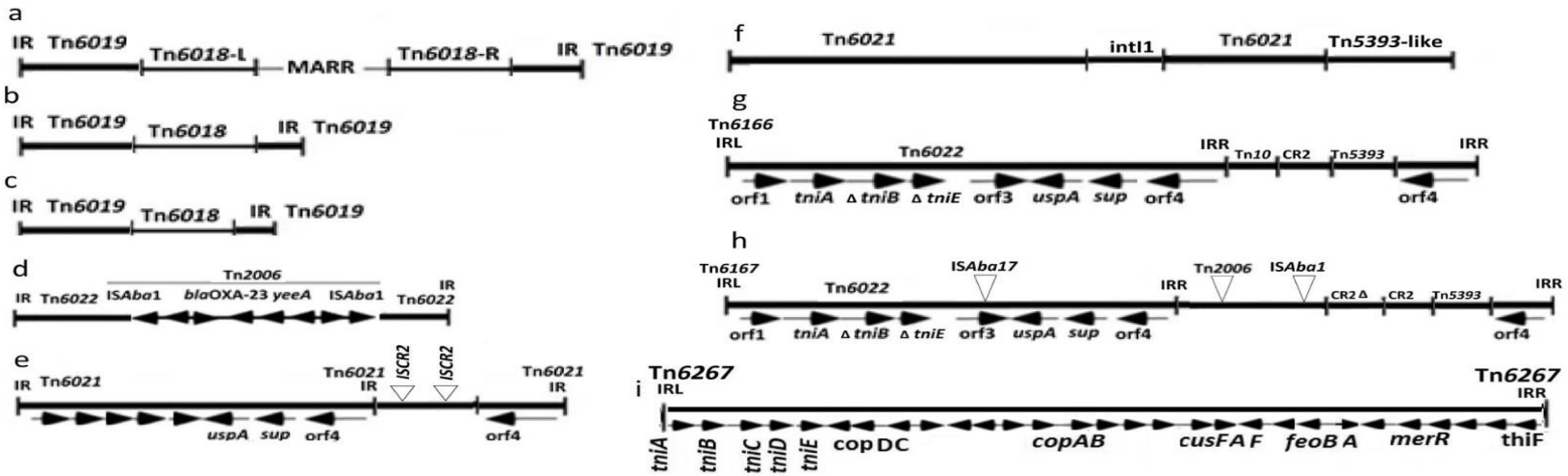

Figure 4.1 Schematic representation of various backbone transposons in AbaR islands.
Common AbaR backbone transposon Tn*6019* (a) with highly variable multiple antibiotic resistance region MARR is bracketed between two directly oriented copies of heavy metal transposon Tn*6018*. Tn*6019* is found represented in AbaR0 strain WM98, AbaR1 strain AYE, AbaR3 strain AB0057, AbaR5 strain 3208, AbaR8 strain D13 clone GCI, AbaR9 strain AB056, AbaR10 strain AB058, AbaR12 strain LUH6013, AbaR13 strain 6015, AbaR15 strain LUH6125, AbaR16 strain LUH7140, AbaR17 strain LUH8592, AbaR18 strain NIPH2713, AbaR19 strain NIPH2554, AbaR20 strain HK302, AbaR21 strain RUH875, Tn*AbaR23* strain A424, AbaR23 strain D81 clone GCI and AbaR24 strain A1 clone GCI. Variant form of Tn*6019* (b) harbouring deletion of MARR represented in AbaR11 strain NIPH470. Truncated transposon Tn*6019* (c) due to internal deletion is represented as a backbone transposon in AbaR2 strain ACICU, AbaR6 strain D2, AbaR7 strain A92, AbaR14 strain LUH5881, AbaR26 strain D30. Transposon Tn*6022* (d) is represented as a backbone transposon in AbaR4 strains ATCC17978, D36, AB0057, AB210, A85 and IOMTU 433. Transposon Tn*6022* carrying Insertion sequence IS*CR2* integrated towards its 3' end (e) is represented in *AbaR4a* (strain LT-3), AbaR4b (strain LT-11), AbaR4c (strain LT-V1), AbaR4d (strain 1656-2) and AbaR4e (strain TCDC-AB0715). Transposon backbone of AbaR22 in strain MDR-ZJ06 (f) formed by two directly oriented copies of Tn*6021.* Transposon Tn*6166* (g) is found in strain RUH134 and is predicted to form the backbone of GI_*comM*_A25 in strain A25 due to their 100% DNA sequence identity. Transposon Tn*6167* (h) appearing as a backbone transposon of AbaR25 in strains BJAB07104, K51-74 and K51-65 and AbaR26 in strain BJAB0868. Transposon Tn*6267* (i) that forms the backbone of GI_*comM*_A92 and GI_*comM*_IS116 possess heavy metal resistance determinants, mainly copper and iron. Schematics based on Krizova *et al.* 2010, Nigro and Hall 2012 and Post *et al.*2010.

4.3.2 PCR mapping of GI_*comM*_A92

Shaikh *et al.* (2009) failed to detect 5'end of GI_*comM*_A92 in strain A92 and the authors assumed that 5' end of GI_*comM*_A92 might have suffered deletion. The sequence analysis of GI_*comM*_IS116 during this work showed the presence of intact five transposition genes towards its 5' end. The GI_*comM*_A92 was examined by PCR mapping in order to determine firstly the extent of similarity between GI_*comM*_IS116 and GI_*comM*_A92 and secondly the presence or absence of transposition genes towards its 5' end before deciding to use the sequence of GI_*comM*_IS116 to represent novel *comM* associated AbaR islands in A92.

The sequence of GI_*comM*_IS116 was used to design PCR primers to map the 5' region of GI_*comM*_A92. The primer pairs used in *in silico* PCR mapping of GI_*comM*_IS116 produced expected amplicon sizes in all instances when used in mapping of GI_*comM*_IS116 (Figure 4.2). Primer pair AbaR1UF and NS13 targeting the amplification of terminal 5' AbaR region produced 2100 bp PCR amplicon from GI_*comM*_IS116 but a smaller amplicon (~1.4 kb) was obtained during PCR in strain A92. The sequence analysis of the 1.4 kb amplicon from A92 showed the presence of 26 bp terminal inverted repeat (IR) at the 5' end of GI_*comM*_A92 indicating the presence of intact island (see appendix A for the sequence of 3' *comM* from strain A92). As predicted by Shaikh *et al.* (2009), a deletion of 616 nucleotides immediately before the inverted repeat of GI_*comM*_A92 towards the 3' *comM* end was detected in strain A92 which resulted in the deletion of five bp direct repeat sequence ACCGC from the 3' end *comM* end. The PCR mapping showed GI_*comM*_A92 as a 35 kb island bounded by terminal IR sequences possessing an array of five transposition genes *tniA, tniB, tniC, tniD* and *tniE* at its 5' end. Since identical PCR fragments were obtained for GI_*comM*_A92 during PCR mapping, the DNA of GI_*comM*_A92 and GI_*comM*_IS116 is therefore assumed to be almost identical. The next section of this chapter is concerned with the nucleotide and amino acid sequence comparison of the backbone transposons.

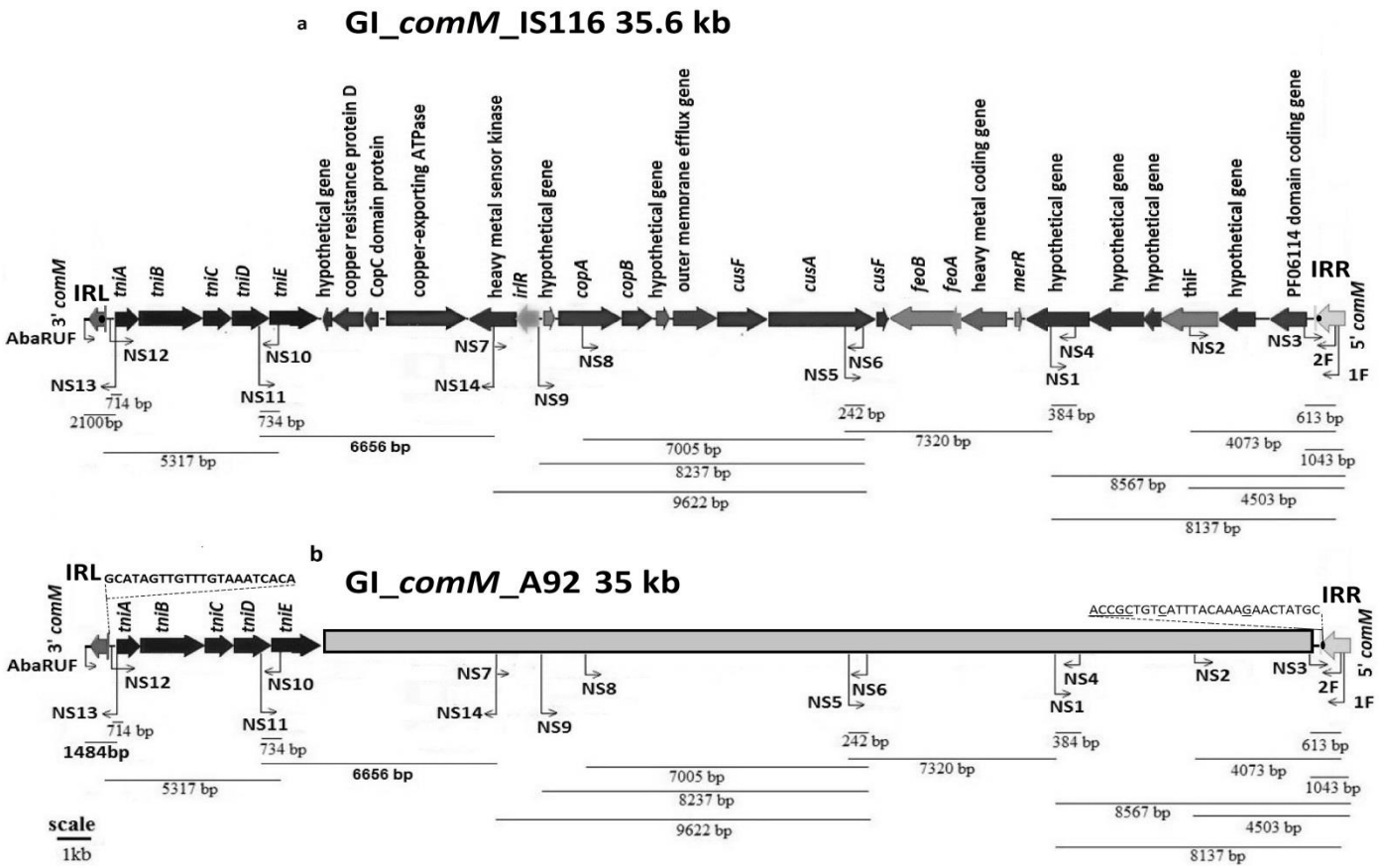

Figure 4.2 PCR mapping of GI_*comM*_A92 and GI_*comM*_IS116.

In silico PCR mapping of 35.6 kb GI_*comM*_IS116 showing genes and determinants with putative function (a). PCR mapping of the 35 kb GI_*comM*_A92 (b) shows intact transposition genes *tniABCDE*. The deletion hypothesized by Shaikh *et al.* (2009) found occurring within 3' *comM* end as evident by the amplification of smaller 1484 bp PCR amplicon by the primer pair NS13 and AbaRUF. Black solid circle at the 3' and 5' *comM* ends represent the five bp direct repeat ACCGC, left and right inverted repeat sequences (IRL and IRR) are represented by vertical lines. Sequence of Tn*6267*_IRL and Tn*6267*_IRR towards the 5' and 3' *comM* is shown in (b).

4.3.3 Phylogenetic relationship between AbaR islands

Nucleotide and amino acid sequences of the transposition genes *tniA, tniB, tniC, tniD and tniE* were extracted from all the AbaR elements listed in the above Table 4.1. The sequences were aligned (whenever available) by clustalW with cut off value of 30% and 500 bootstrap replicates were used for analysis using MEGA6. Strains carrying truncated or interrupted genes were excluded from the trees of genes.

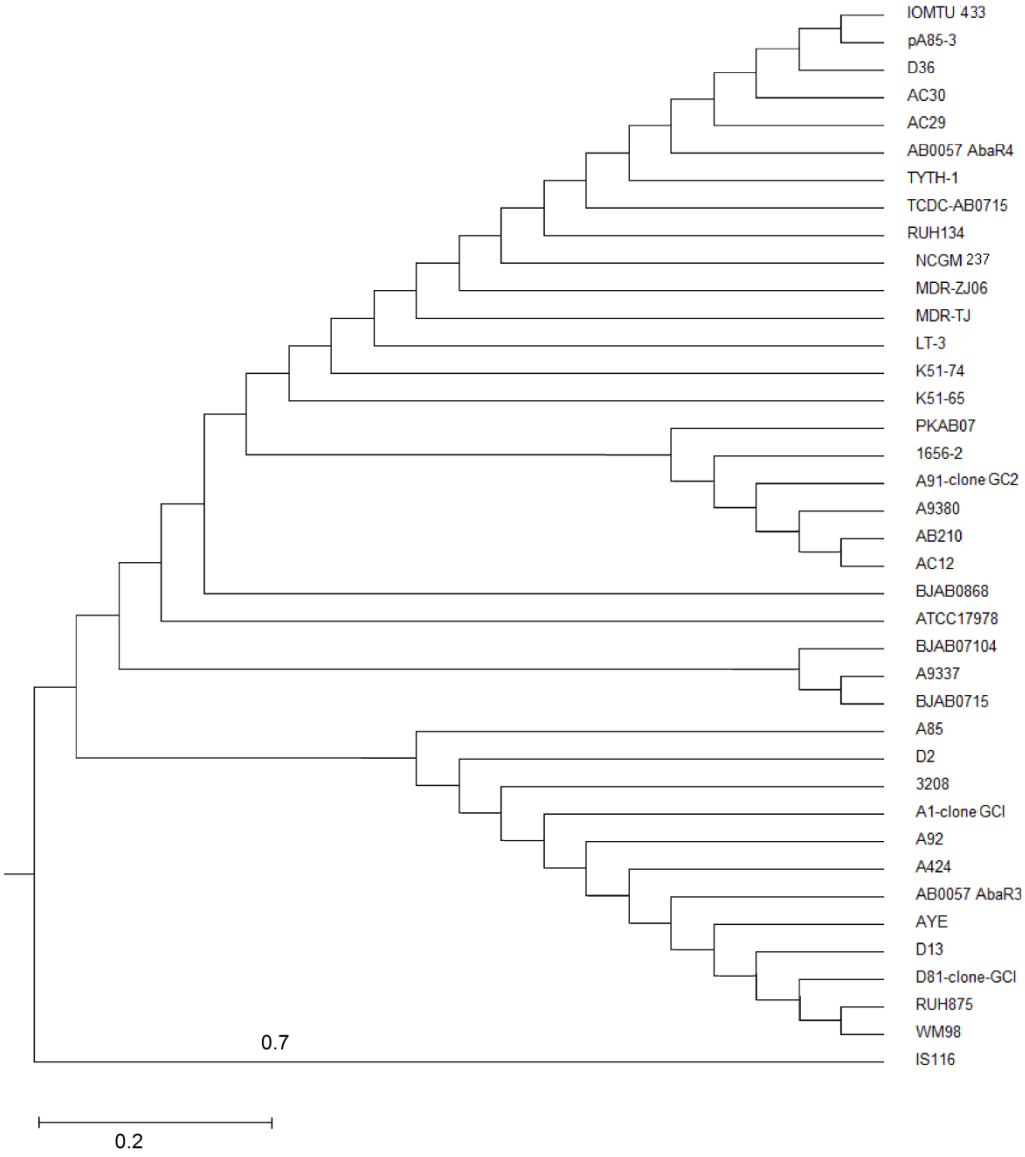

Figure 4.3 Phylogenetic tree constructed using *tniA* nucleotide sequence from AbaR islands.

The nucleotide sequence of *tniA* gene was used to construct the tree. The tree was inferred using the Neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site.

The *tniA* nucleotide sequence of 39 AbaR islands were aligned a stretch of 931 bp *tniA* sequence was fed into MEGA 6 for tree construction. The strain IS116 was placed separately from the rest of the strains in the tree constructed using *tniA* nucleotide sequence from AbaR islands. All the AbaR islands carrying Tn*6019* as their backbone transposon (representing AbaR3 islands) were found clustered in one branch (Figure 4.3). The AbaR islands possessing Tn*6021*, Tn*6022* and Tn*6166* backbone transposons (representing AbaR4-like islands) were found to cluster separately from AbaR3 group. The strain BJABO7104 carrying AbaR25 was grouped with A9337 (Tn*6022* backbone) and BJAB07104 (Tn*6167* backbone) between AbaR3 and AbaR4 cluster. The branch containing AbaR4-like island appeared diverse and contained various sub-branches. The strains ATCC17978 and PKAB07 were grouped separately within the AbaR4 cluster indicating the unique feature of their AbaR4-like island.

High level of amino acid sequence identity was observed for TniA protein from 38 AbaR elements (Figure 4.4). A stretch of 290 amino acid sequence was retrieved from the multiple sequence alignment which was then fed into MEGA 6 for phylogenetic analysis. Of the 38 strains analysed, 36 strains were found clustered in a distinct branch in the phylogenetic tree. TniA protein sequence from AbaR0 in strain WM98 was found to be distinct from all the tested strains. The strain IS116 demonstrated its uniqueness by placing itself separately from the rest of the strains.

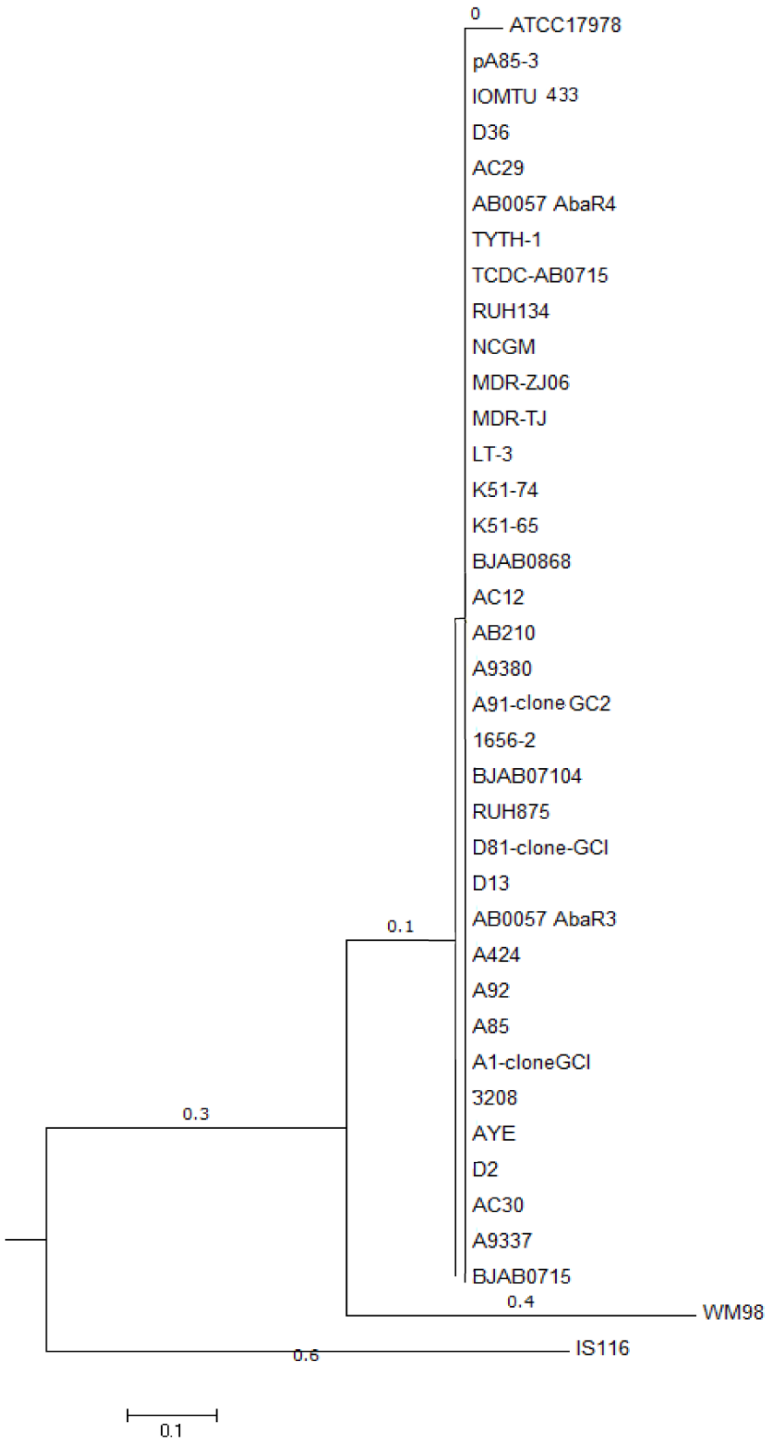

Figure 4.4 Phylogenetic tree constructed using *tniA* amino acid sequence from AbaR islands.

The amino acid sequence of *tniA* gene was used to construct the tree. The tree was inferred using the Neighbour-joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary are in the units of the number of amino acid substitutions per site.

A stretch of 2064 bp *tniB* nucleotide sequence retrieved from the multiple sequence alignment was used for phylogenetic analysis. The tree constructed using the *tniB* nucleotide sequence (Figure 4.5) was found to have three major branches. The first branch which was placed distinctly separate from rest of the branches belonged to IS116. The second branch belonged to the strains BJAB0715, A9337 and ATCC17978.

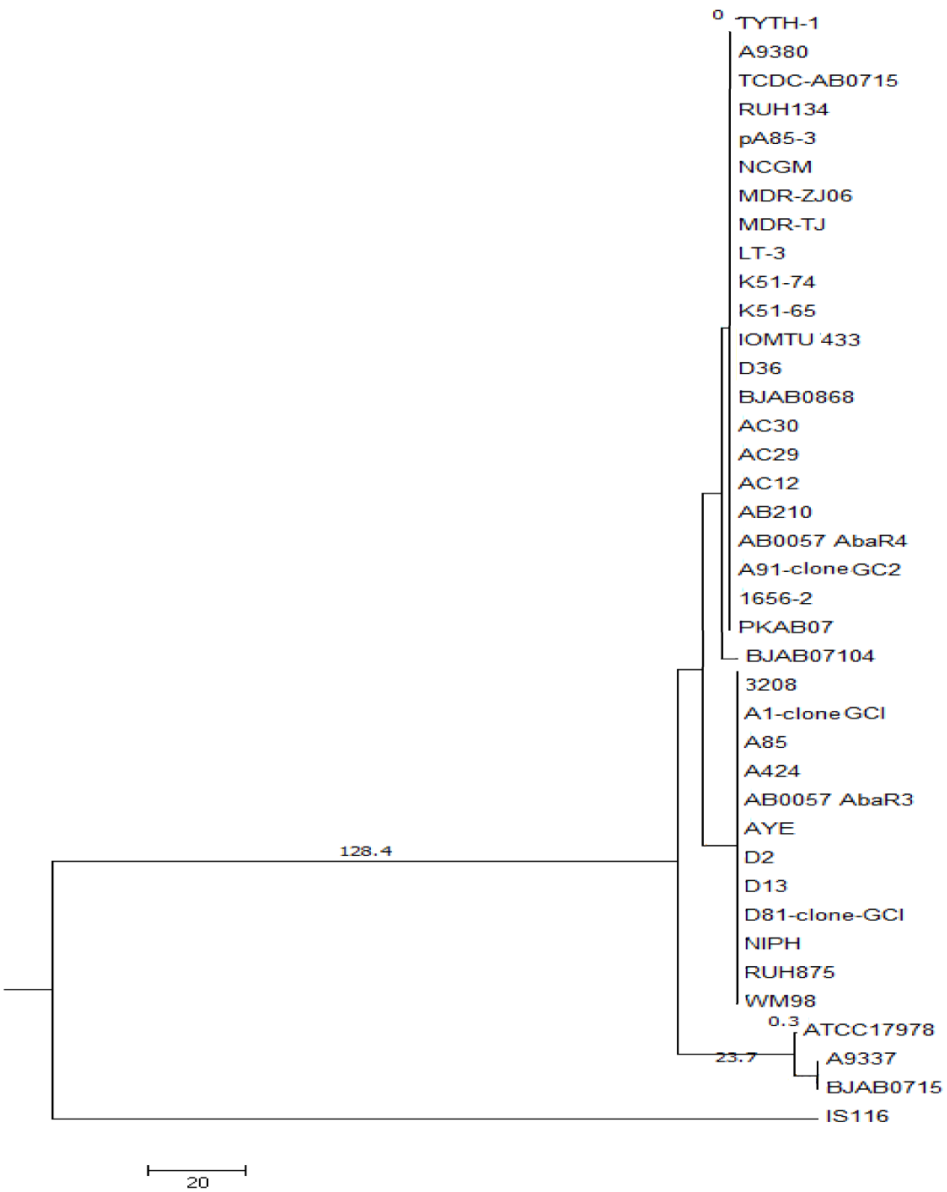

Figure 4.5 Phylogenetic tree constructed using *tniB* nucleotide sequence from AbaR islands

The nucleotide sequence of *tniB* gene was used to construct the tree. The tree was inferred using the Neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site.

The *tniB* nucleotide sequence of these three strains was very similar but distinctly different from the other tested strains. The third large branch collectively possessed strains carrying AbaR3 and AbaR4-like islands with a clear sub-branching leading to AbaR3 and AbaR4 carrying strains. The TniB amino sequences of the 38 strains were aligned and a stretch of 653 amino acid long TniB protein sequence was obtained for use in phylogenetic analysis. Except from the strain IS116, the TniB amino acid sequence of all the tested strains was found highly similar. The TniB amino acid tree in Figure 4.6 can be seen divided into two major branches. One branch carries only one strain IS116 and the other branch carries all the tested 37 strains.

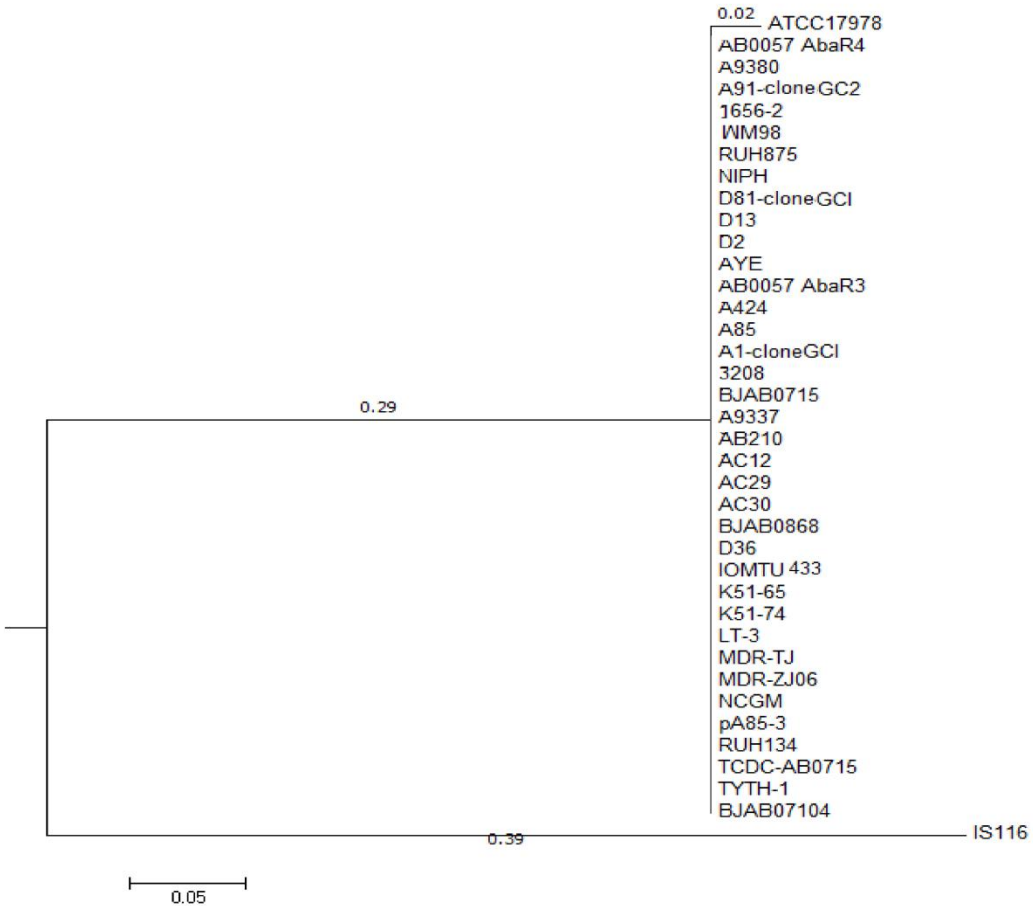

Figure 4.6 Phylogenetic tree constructed using *tniB* amino acid sequence from AbaR islands

The amino acid sequence of TniB was used to construct the tree. The tree was inferred using the Neighbour-joining method and is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of amino acid substitutions per site.

The transposition genes of some of the AbaR elements were found truncated due to integration, deletion and genetic rearrangement within their AbaR host. In order to

accommodate the strain carrying partial or truncated transposition genes, the nucleotide and amino acid sequences of *tniA*, *tniB*, *tniC*, *tniD* and *tniE* were artificially joined to create a concatenated *tniABCDE* (nucleotide) and TniABCDE (amino acid).

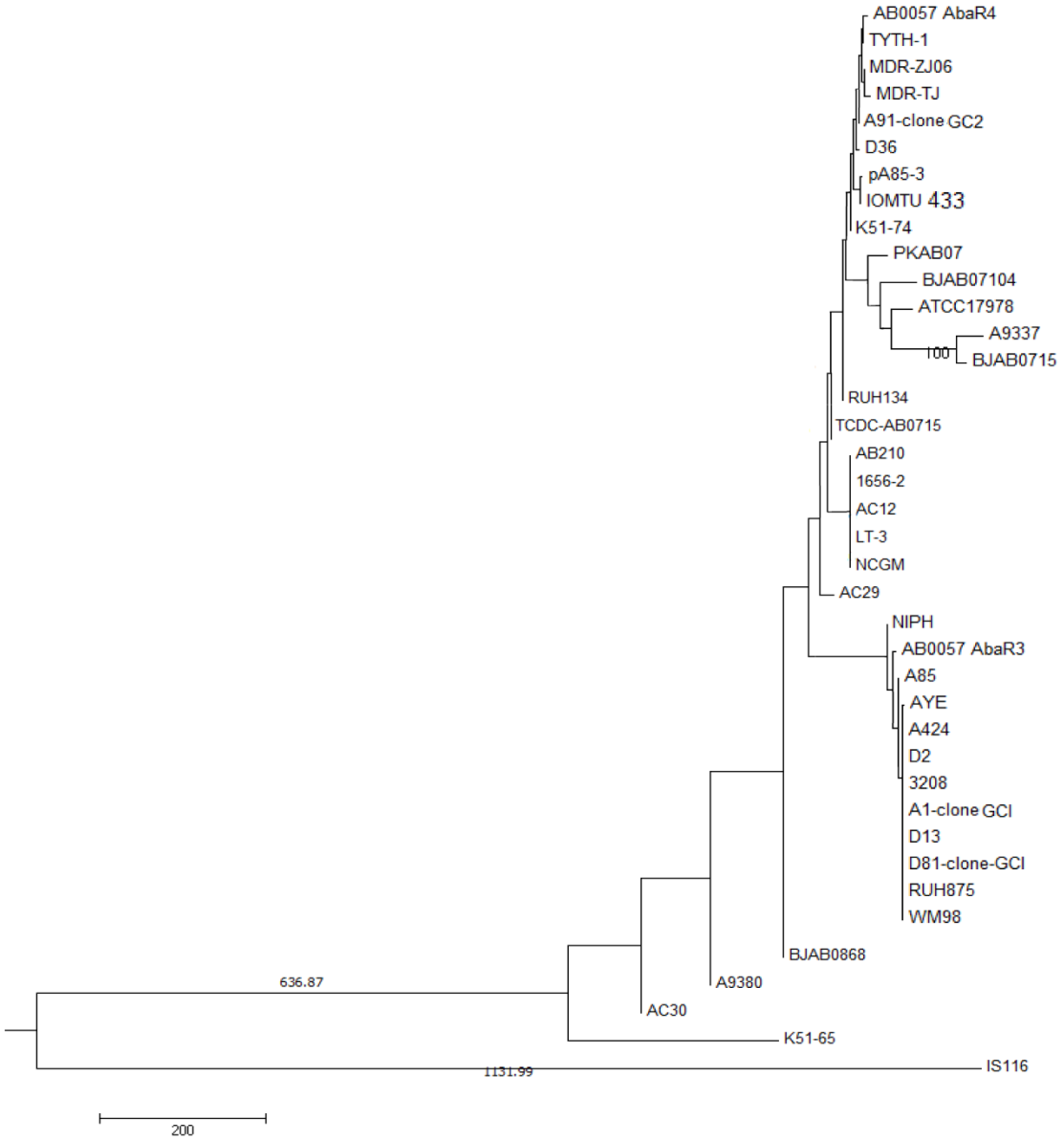

Figure 4.7 Phylogenetic tree constructed using concatenated *tniABCDE* nucleotide sequence from AbaR islands.
The nucleotide sequence of concatenated *tniABCDE* was used to construct the tree. The tree was inferred using the Neighbour-joining method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site.
The concatenated sequences of representative AbaR elements were aligned and tree was constructed for phylogenetic analysis.

A stretch of 6763 bp nucleotide sequence from concatenated *tniABCDE* (excluding any intergenic regions) was used for tree construction using MEGA 6 (Figure 4.7). The tree branches very early creating two major sub-branches; one belongs to IS116 and second one belonging to remaining all 38 strains. However, the second branch further splits to form 5 distinct groups. After diversifying into Tn*6267* transposon (represented in strain IS116), the putative ancestral transposon of AbaR elements appeared to have evolved into Tn*6166* and Tn*6167*-like transposons (represented in strains K51-65, AC30, A9380 and BJAB0868) before further evolving into transposons currently represented in AbaR3 and AbaR4-like elements. Further diversification of Tn*6019* is not observed however; transposons forming backbone of AbaR4-like islands appear to be evolving in many dimensions as apparent from the emergence of sub-groups within the AbaR4 branch in the above phylogenetic tree (Figure 4.7).

A stretch of 2095 amino acid from concatenated TniABCDE was obtained for phylogenetic tree construction after the multiple sequence alignment of concatenated TniABCDE from 37 representative strains. The tree of concatenated TniABCDE (Figure 4.8) shows the early diversification of unknown putative ancestral transposon to give rise to AbaR backbone transposon in strain IS116 and AbaR backbone transposon in AbaR3 and AbaR4-like islands known today. The branch leading to the transposons in AbaR3 and AbaR4 formed sub-branches clearly demarcating the emergence of Tn*6019* transposons in AbaR3 (like in strains WM98 and A424).

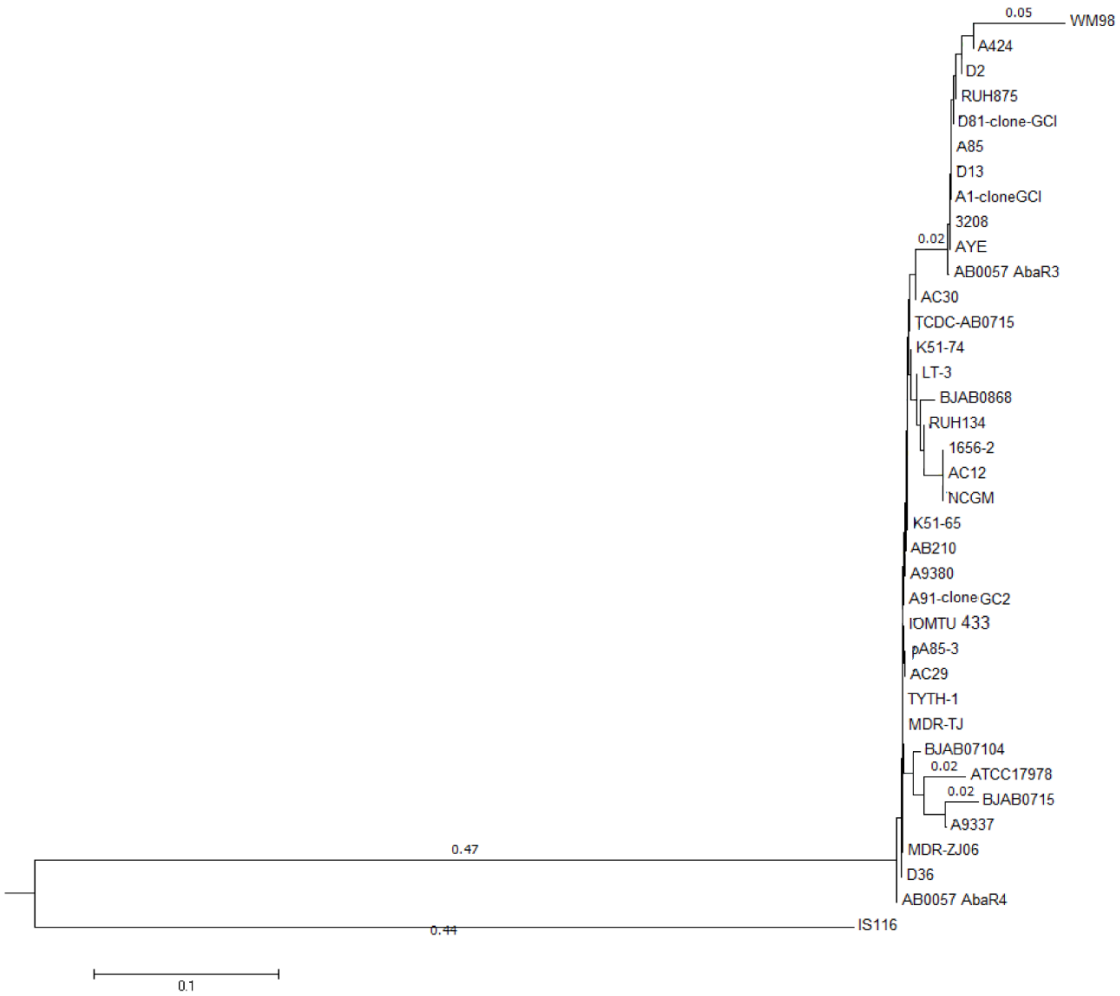

Figure 4.8 Phylogenetic tree constructed using concatenated TniABCDE amino acid sequence from AbaR islands.
The amino acid sequence of TniA, TniB, TniC, TniD and TniE was artificially joined to create a concatenated TniABCDE protein. The tree was inferred using the Neighbour-joining method and is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of amino acid substitutions per site. The tree was constructed in MEGA 6.

Various AbaR4-like islands carry variant forms of backbone transposons like Tn*6021*, Tn*6022*, Tn*6166* and Tn*6167*. The sub-branching of the cluster of strains carrying AbaR4-like islands clearly shows the diversification of their backbone transposons giving rise to novel backbone transposons like the one observed in AbaR25 in strain BJAB0715. Compared to AbaR4 forming backbone transposons, very little diversification was observed among the AbaR3 backbone forming transposon Tn*6019*.

4.4 Discussion

4.4.1 Survey of AbaR islands

This study was set out to establish the phylogenetic relationship between diverse appearing AbaR islands and aiming to establish the lineages of these elements in *A. baumannii*. Genbank database was searched using Tn*AbaR23* sequence from strain A424 as a query sequence. A total of 51 AbaR islands were reported in the published literature with sequence available in the Genbank database. The AbaR-islands or strains whose sequence is not available in the Genbank database are excluded from this study. In this study, by comparative sequence analysis, seven previously unreported AbaR elements have been identified. Using comparative sequence analysis, the constituent backbone transposon was identified in all 57 AbaR-like islands. For AbaR27 in strain BJAB0715, Zhu *et al.* (2013) have not identified the backbone transposon, therefore, in this study the AbaR27 island backbone has been considered as not known [NK].

The *comM*-associated sequence in strain A92 (Shaikh *et al.* 2008) was found almost identical (based on PCR mapping) to the *comM* associated sequence in strain IS116. The backbone transposon of AbaR island in strains A92 and IS116 (this study) was subsequently assigned a transposon number Tn*6267*. The comparative sequence analysis showed AbaR island in strain A25 (Shaikh *et al* 2008) to have a backbone transposon Tn*6166*; the AbaR island in strain A25 was subsequently given an interim name GI_*comM*_A25. During the survey, previously unreported seven AbaR islands were identified in strains IS116, MDR-TJ, PKAB07, IOMTU 433, A30, AC29 and AC12 and the AbaR islands in these strains were given interim name GI_*comM*_IS116, GI_MDR-TJ, GI_PKAB07, GI_IOMTU 433, GI_A30, GI_AC29 and GI_AC12 respectively. The *in silico* mapping of GI_*comM*_IS116 and PCR mapping of GI_*comM*_A92 revealed the presence 35.6 kb and 35 kb heavy metal resistance determinants (mainly copper resistance determinants and genes associated with iron acquisition) carrying AbaR-like islands build on the backbone of novel transposon Tn*6267.*

4.4.2 Lineage of AbaR islands

The present study was conducted to examine the backbone transposons in AbaR islands aiming to establish a phylogenetic relationship between diverse AbaR islands known to-date. The phylogenetic trees created using the nucleotide and protein sequences of *tniA*, *tniB*, *tniC*, *tniD* and *tniE* genes from representative AbaR backbone transposons shows that AbaR islands in *A. baumannii* have been evolving and diversifying. The novel AbaR island GI_*comM*_IS116 in MDR strain IS116 isolated from Iraq appears to have diversified early from the, yet unknown, transposon ancestral to AbaR elements known today. The sequence analysis of the GI_*comM*_IS116 backbone transposon Tn*6267* strongly suggests GI_*comM*_IS116 to be a distant and distinct family of AbaR elements. However, no evidence was found that indicated the further evolution and diversification of GI_*comM*_IS116-like islands since this island was represented in only two strains A92 and IS116. In future, more AbaR sequence data is anticipated to be available that will allow to trace the diversification of, if any, GI_*comM*_IS116 islands. But for now, GI_*comM*_IS116 appears to have entered a silent phase in terms of evolution, which is most likely might not the case. As for now, GI_*comM*_IS116 stands as a distant family of AbaR elements in *A. baumannii.*

The phylogenetic analysis shows that the unknown putative ancestral AbaR transposon evolved to form two distinct branches, one of the branches is represented by GI_*comM*_IS116 while the other branch further evolved to form a range of transposons like Tn*6019*, Tn*6166*, Tn*6167*, Tn*6021* and Tn*6022*. Except from Tn*6019*, all of these transposons are found as a backbone in AbaR4-like elements. These AbaR4 elements represent around 52% of all known AbaR elements in *A. baumannii*. It was interesting to observe clustering of AbaR islands backbone transposons like Tn*6166*, Tn*6021*, Tn*6022* and Tn*6167* in the concatenated TniABCDE nucleotide and amino acid tree, which suggests their close but distinct relatedness, most probably due to recent evolution. Consistent with the analysis of Hamidian and Hall (2011), this phylogenetic analysis convincingly indicates that AbaR4 backbone transposons, and by extension, AbaR4-like elements form a second lineage of AbaR elements. The phylogenetic tree

also shows an extensive branching and sub-branching within AbaR4 elements exhibiting their evolution on-the-go.

Undoubtedly, AbaR4-like elements are evolving and diversifying creating a wide range of diverse AbaR elements like AbaR islands represented in strains A9337 (Tn*6022*-like), BJAB07104 (Tn*6167*-like), ATCC17978 (Tn*6021*-like), and PKAB07 (Tn*6166*-like) and last but not least strain BJAB07150 (AbaR27). Along with their capabilities of rapid evolution, it is possible that the AbaR4-like elements are capable of disseminating their cargo resistance determinants across the species. The recent identification of novel AbaR4-like island in MDR *A. baumannii* from China (Zhu *et al.* 2013) and non-*baumannii Acinetobacter* species from South Korea (Kim and Ko 2015) strengthens the idea that diverse AbaR4 elements are highly mobile. This makes AbaR4 elements one of the highly concerning mobile elements in terms of emergence and dissemination of antimicrobial resistance.

Except from AbaR4, the first AbaR elements in *A. baumannii* were identified in MDR clinical *A. baumannii* strains. All these AbaR elements were found to be built on Tn*6019* backbone. The work of Krizova *et al.* (2011) elegantly shows the evolution and diversification of AbaR3-like elements, however, during the phylogenetic analysis it was interestingly noted that unlike AbaR4-like elements, AbaR3-like elements were showing less diversification and therefore less evolution. It is however important to recall that much of diversity among AbaR3-like elements is observed within their multiple antibiotic resistance region (MARR) and that multiple events of intermolecular recombination have been reported within this MARR region of AbaR3-like elements. Although, compared to AbaR4-like elements, AbaR3-like elements appear quiescent in-terms of evolution; it is likely that AbaR3-like elements are actively involved in disseminating the antimicrobial resistance across the species. Consistent with the observation of Hamidian and Hall (2011), this phylogenetic analysis have established Tn*6019* and, by extension, AbaR3-like islands as a discrete family of AbaR islands.

This study was based on the nucleotide and amino acid sequence comparison of only five transposition genes from AbaR backbone transposons. Therefore these results

need to be interpreted with caution. As more and more sequence data of *A. baumannii* becomes available, a clear picture of the families of AbaR islands with discrete lineages is expected to emerge. However, this study provides some dependable conclusions that *A. baumannii* AbaR islands might have diverged from a common yet unknown ancestral transposon.

Based on the phylogenetic analysis of transposon backbone gene, it is hypothesized that AbaR islands have diversified to form three major lineages. The first lineage is represented by AbaR3 islands built on backbone of Tn*6019* and around 45% of MDR *A. baumannii* possess variant forms of this island. The second lineage is represented by AbaR4-like islands built on the backbone of diverse transposons like Tn*6022*, Tn*6166*, Tn*6167* and Tn*6021*. The AbaR4-like islands are the most abundant islands and are found represented in 50% of MDR *A. baumannii* genome. The novel GI_*comM*_IS116-like island represented in strains IS116 and A92 represents another distant lineage of AbaR-elements. Since AbaR4-like elements were found to be diversifying to form their variant versions, in agreement with Zhu *et al.* (2013), I have described AbaR27 as a variant form of AbaR4-like islands and but I have not considered it as a major lineage of AbaR islands. Since AbaR4-like islands appear to be diversifying rapidly, it will be interesting to watch the evolutionary journey of AbaR4-like elements and how they drive the emergence and dissemination of antimicrobial resistance in *A. baumannii.*

5 Plasticity of AbaR islands

5.1 Chapter overview

The survey of ***A****cinetobacter* ***ba****umannii* **r**esistance islands (AbaR) has shown that the genome of MDR *A. baumannii* strains possesses at least one type of these AbaR islands. Multiple events of genetic rearrangements have been reported in diverse AbaR islands making them unstable features in the genome of *A. baumannii* (Krizova *et al.* 2011). In the introduction chapter, I have briefly described the presence of 48.3 kb AbaR island called Tn*AbaR23* in A424 which was found inserted in a site and orientation-specific manner within the chromosomal *comM* gene (Kochar *et al.* 2012). Deletion and intramolecular genetic rearrangements mediated by identical copies of insertion sequences and transposons within several AbaR islands have been frequently reported and this phenomenon is described as "central" to the evolution and diversification of AbaR islands (Krizova *et al.* 2011, Saule *et al.* 2013, Harmer *et al.* 2014). During the PCR analysis of the Tn*AbaR23*, a possible deletion of a large region internal to Tn*AbaR23* was noticed. The deletion was found occurring at **m**ultiple-**a**ntibiotic **r**esistance **r**egion or MARR. This MARR is a region internal to Tn*AbaR23* and is bracketed by two identical copies of heavy metal transposon Tn*6018*.

5.2 Aim

In this chapter, I will be investigating the stability of Tn*AbaR23* island in *in vitro* growth conditions. Using PCR based semi-quantitative assays I will be studying the rate of deletion and loss of the region/s internal to MARR in a population of A424 strain.

5.3 Results

5.3.1 PCR detection of spontaneous deletion within Tn*AbaR23*

The primer pair PR1766 and PR1767 spanning the 33 kb compound transposon Tn*6018*-MARR-Tn*6018* was found to produce 4.7 kb PCR product when the genomic DNA or gDNA of A424 was used as a PCR template (Figure 5.1a). It was apparent that a deletion or genetic rearrangement within Tn*6018*-MARR-Tn*6018* had occurred which had shortened the distance between the above primer pair. Same primer pair was used to probe the genome of another MDR strain AYE which possessed 86 kb AbaR1 island within the chromosomal *comM* gene.

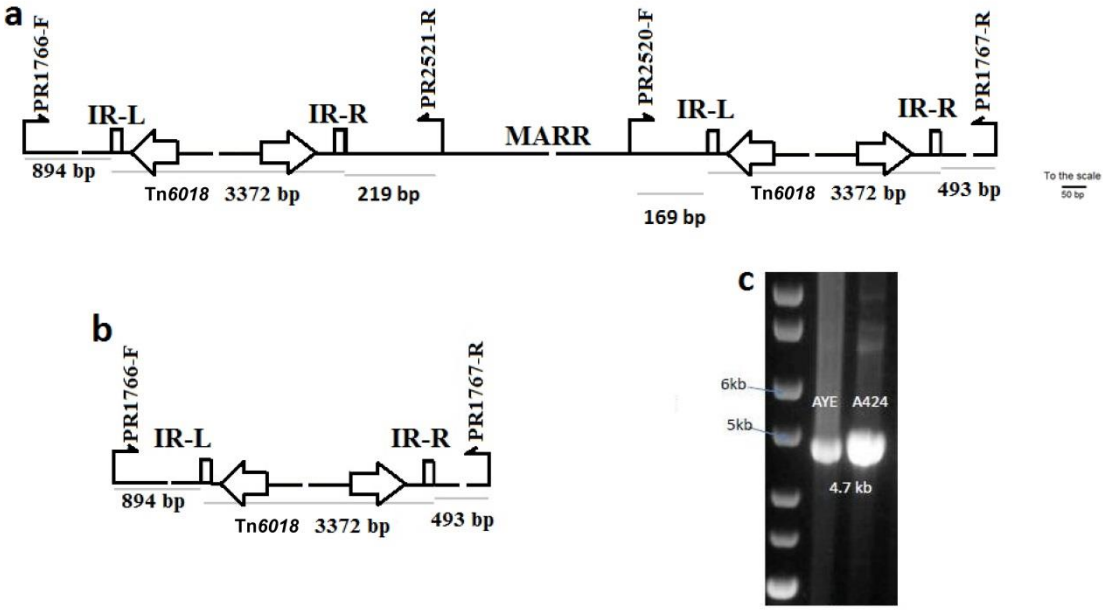

Figure 5.1 Detection of Tn*6018*-MARR deletion in AYE and A424
The primer pair PR1766 and PR1767 annealing outside Tn*6018*-MARR-Tn*6018* (a) amplifying 4.7kb PCR product in *A. baumannii* strains AYE and A424 (c) upon the deletion of Tn*6018*-MARR (b). The MARR region in (a) and Tn*6018* in (b) are not drawn to the scale.

Surprisingly, the primer pair amplified 4.7 kb PCR product from the gDNA of strain AYE (Figure 5.1c) strongly indicating that the deletion within Tn*6018*-MARR-Tn*6018* was a common phenomenon in Tn*AbaR23* and AbaR1 and probably in other AbaR islands. Sequence analysis of AbaR1 and Tn*AbaR23* showed that the primer pair PR1766 and PR1767 would produce a 4.7 kb PCR product only upon the deletion of regions internal

to the compound transposon Tn*6018*-MARR-Tn*6018* (Figure 5.1b). The gDNA of A424 was subsequently probed to check the possible existence of a double stranded circular element that would have excised from Tn*6018*-MARR-Tn*6018*.

5.4 Detection of double stranded circular element excised from Tn*6018*-MARR-Tn*6018*

The outward facing primer pair PR2521 and PR2520 (Figure 5.1a) annealing within MARR were designed to detect the junction of a possible double stranded circular element that would have formed post spontaneous deletion in Tn*AbaR23*. This primer pair produced a characteristic 3760 bp PCR product from A424 gDNA (Figure 5.2b) indicating that Tn*6018*_MARR was being excised as a double stranded circular element (Figure 5.2c). The sequence analysis of the junction of 3.7 kb circular DNA (see Appendix A for the sequence of circular junction) showed that this element possessed intact MARR and transposon Tn*6018* along with the left and right inverted repeat sequences of Tn*6018*. The second copy of Tn*6018* was left *in situ* as a remnant of the compound transposon Tn*6018*-MARR-Tn*6018* within now much truncated Tn*AbaR23*ΔTn*6018*-MARR (Figure 5.2a).

Along with determinants for various antibiotics, the MARR region in Tn*AbaR23* carries determinant conferring sulfamethoxazole resistance. In the event of deletion and subsequent loss of Tn*6018*-MARR from the cell, the resulting Tn*6018*-MARR deleted strain would become sensitive to sulfamethoxazole. Experiment was subsequently designed to isolates the sulfamethoxazole susceptible spontaneous mutant of A424 strain.

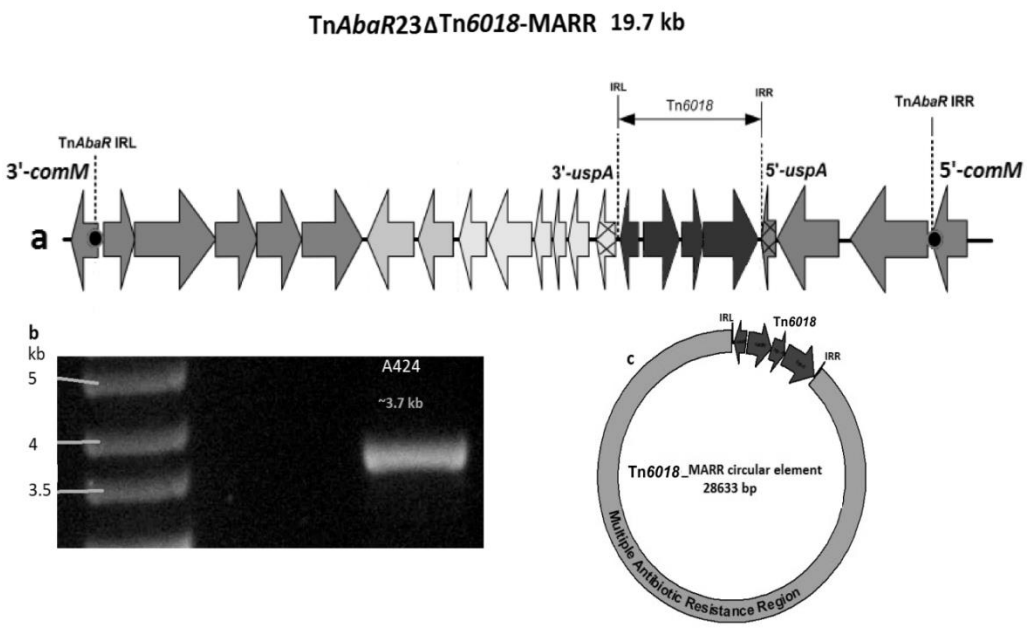

Figure 5.2 Detection of circular Tn*6018*-MARR element in A424
Schematics representing truncated Tn*AbaR23*ΔTn*6018*-MARR post deletion of Tn*6018*-MARR in spontaneous mutants (a), the Tn*6018*-MARR circular DNA (c) and 3.7 kb junction of circular DNA amplified from A424 by primer pair PR2520 and PR2521 (b). The presence of circular Tn*6018*-MARR element in A424 was predicted based on the detection of 3.7 kb the circular junction.

5.5 Isolation of the Tn*6018*-MARR deleted spontaneous mutants

To select the sulfamethoxazole sensitive spontaneous mutant from a population of A424 strain, appropriate dilution of the overnight culture of A424 was plated on Muller-Hinton agar (MHA). The colonies from MHA were parallel patched first on MHA and then on MHA supplemented with 40 µg/ml sulfamethoxazole. The sulfamethoxazole sensitive colonies were selected from MHA plates and were verified for the loss of Tn*6018*-MARR.

5.5.1 Determining the proportion of A424 derived cells that have lost Tn*6018*-MARR

In three independent experiments, a total of 1200 A424 colonies were patched on MHA and on MHA supplemented with 40 µg/ml sulfamethoxazole. A total of four

sulfamethoxazole susceptible colonies were obtained. The proportion of cells lacking Tn*6018*-MARR element was found to be around 1 in 300 cells.

5.5.2 Verification of Tn*6018*-MARR deleted spontaneous mutants

The loss of Tn*6018*-MARR in sulfamethoxazole sensitive colonies was verified by amplifying the empty region previously occupied by Tn*6018*-MARR. The parent strain A424 was used as a positive control and the strain DCOA14 that lack Tn*AbaR23* was used as a negative control.

The PCR reaction for the positive and negative controls contained 20 ng of gDNA whereas the test samples contained 10 ng gDNA of putative spontaneous mutants (Figure 5.3b). The faint 4.7 kb band observed in A424 indicates that only a small proportion of cells in the entire population might harbour Tn*6018*-MARR deletion within their Tn*AbaR23*. To check whether the excised Tn*6018*-MARR has integrated elsewhere in the genome, the sulfamethoxazole sensitive colonies were tested for the presence of MARR associated *aadA1* gene (Figure 5.3c). The gDNA of A424 and AbaR1 carrying MDR strain AYE was used as a positive control. The negative *aadA1* PCR in all tested putative mutants confirmed that upon the excision from its native site, the Tn*6018*-MARR element did not re-integrated elsewhere in the genome of the spontaneous mutants and was lost entirely from the cell.

The Pulsed Field Gel Electrophoresis or PFGE profile of spontaneous mutants (Figure 5.3a) was also consistent with the deletion of Tn*6018*-MARR. The strain DCOA14 which lack Tn*AbaR23* was used in the first lanes of gels containing *NotI* and *SfiI* digested high molecular gDNA. The spontaneous mutant specific bands that appeared in *NotI* and *SfiI* digestion are indicated by white arrows. All four independent spontaneous mutants exhibited indistinguishable PFGE profile and band discrepancy was not observed in all four tested mutants.

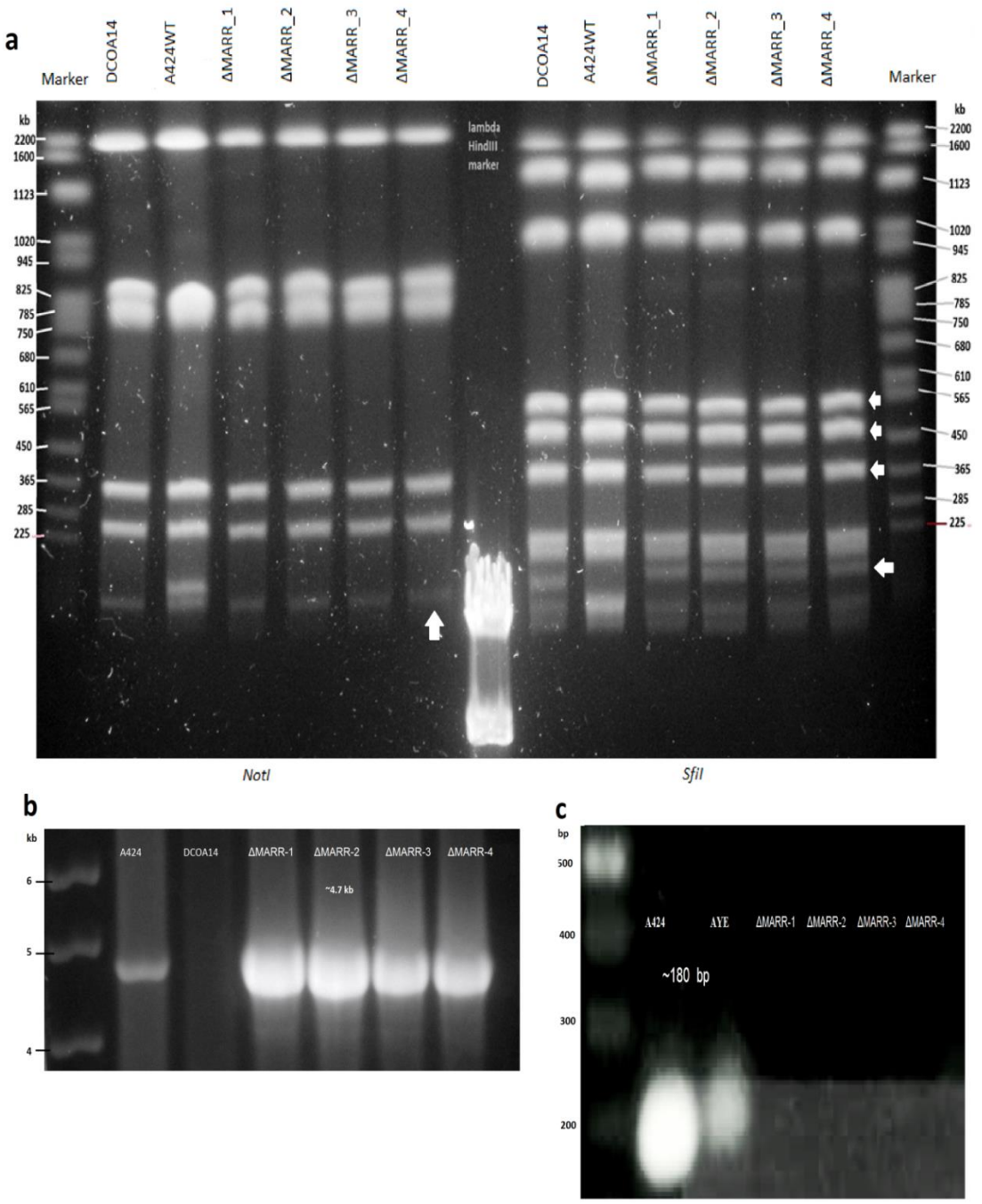

Figure 5.3 PFGE of Tn*6018*-MARR deleted spontaneous mutants
The PFGE profile of four independent Tn*6018*-MARR deleted spontaneous mutants ΔMARR-1, ΔMARR-2, ΔMARR-3 and ΔMARR-4 was indistinguishable indicating them to be isogenic. The arrows indicate the observed *NotI* and *SfiI* profile difference (a). PCR amplification of the Tn*6018*-MARR deleted empty region in spontaneous mutants and A424 wild type (b). PCR amplification of MARR associated *aadA1* gene (c), negative PCR in all the sulfamethoxazole sensitive colonies shows the absence of MARR associated *aadA1* gene in their genome.

The PFGE band profile of the spontaneous mutants remained same in repeated sub culture showing that the genetic configuration observed in the spontaneous mutants was stable and was unlikely to alter by further genetic rearrangements. Four independent spontaneous mutants ΔMARR-1, ΔMARR-2, ΔMARR-3 and ΔMARR-4 were archived in the laboratory collection as KR3324, KR3325, KR3326 and KR3327 respectively (see strain table in method section).

5.6 Determining the proportion of cells carrying circular Tn*6018*-MARR

A PCR based semi-quantitative method was used to detect the proportion of A424 cells carrying Tn*6018*-MARR circular element. Primer pair PR2520 and PR2521 was used to detect the 3.7 kb junction of the double stranded circular DNA. A stock solution containing 2 µg of A424 gDNA per 5 µL of PCR grade water was prepared. A series of 1/10 dilutions of the gDNA stock solution was set up to obtain dilutions with 200 ng/5 µL, 20 ng/5 µL , 2 ng/5 µL, 0.2 ng/5 µL, 0.02 ng/5 µL, and 2 pg/5 µL water. The sensitivity PCR was set up using 5 µl/reaction of the A424 gDNA template at various dilutions (Figure 5.4).

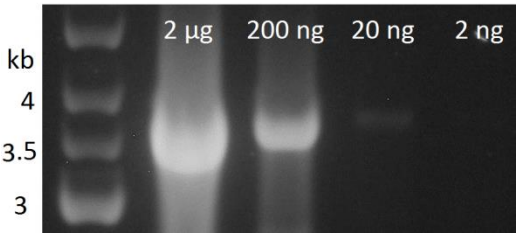

Figure 5.4 Circular junction detection sensitivity assay
The circular junction detection sensitivity assay was performed in A424 wild by using the primer pair PR2520 and PR2521 to amplify the 3.7 kb junction of circular Tn*6018*-MARR element. The cycling condition was adjusted to 45 cycles. Entire PCR product (20µl) was run on gel. Each PCR reaction contains indicated amount of A424 gDNA. The PCR detected the circular element from up to 2 ng of A424 gDNA (very faint band was visible on the gel). The circular junction was not detected in sample containing 0.2 ng or less gDNA (not shown on this gel).

The junction of circular Tn*6018*-MARR element was detected in the sensitivity PCR when the reaction contained up to 2 ng of gDNA (Figure 5.4). The visualization of the

circular junction from the PCR reactions containing 0.2 ng and less A424 gDNA was beyond the detection limit of the agarose gel. The size of A424 genome is ~4 MB. In 1 ng of gDNA, there are roughly 10^5 copies of A424 chromosomal DNA. The gDNA of A424 contains a mixture of chromosomes carrying intact and truncated Tn*AbaR23*. In this sense a sub population of A424 chromosomes with intact Tn*AbaR23* serves as a non-target chromosome. The sensitivity assay started to detect the junction of circular Tn*6018*-MARR element from 2 ng of A424 gDNA implying that in a population of ~10^5 cells, around 100-1000 cells possess circular Tn*6018*-MARR element.

5.7 Determining the proportion of cells harboring Tn*6018*-MARR deletion within Tn*AbaR23*

A PCR based semi-quantitative method described earlier was used to detect the proportion of cells harbouring Tn*6018*-MARR deletion within Tn*AbaR23*. For the sensitivity assay, primer pair PR1766 and PR1767 was used to amplify the 4.7 kb empty region from Tn*6018*-MARR deleted spontaneous mutant ΔMARR-3 gDNA. The first set of PCR was done with samples containing gDNA in various dilutions. To test the sensitivity of the assay, the second set of PCR was done by normalizing the reactions with non-target chromosomal DNA from a Tn*6018*-MARR-Tn*6018* lacking strain DCOA14 ensuring that all the reactions contain a total mass of 2 µg DNA.

Since the entire population of ΔMARR-3 possess Tn*6018*-MARR deletion in their chromosome, the PCR assay easily amplified the empty region when around 100 copies of chromosomes (0.002 ng) were used in the PCR reaction as a template (Figure 5.5a). However, in case of PCR reactions normalized with around 10^8 non-target chromosomal DNA of DCOA14 (Figure 5.5b), a 10 fold decrease in PCR sensitivity was observed and the presence of empty region in ΔMARR-3 was detected only when 00.2 ng or more ΔMARR-3 gDNA (10^3 chromosomal DNA) was used as a template. The sensitivity assay showed that the PCR starts to detect the Tn*6018*-MARR deletion when the reaction contained 100 – 1000 copies of target chromosomes harbouring the deletion.

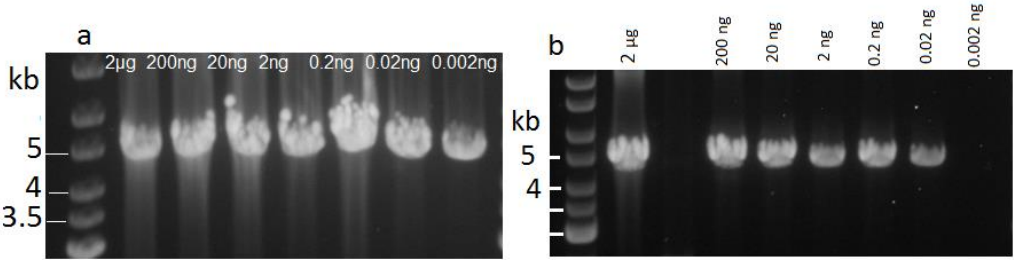

Figure 5.5 Deletion detection sensitivity assay
Primer pair PR1766 and PR1767 was used to amplify 4.7 kb empty MARR region from the genome of spontaneous mutant ΔMARR-3. The cycling condition was adjusted to 45 cycles and entire PCR product (20 µl) was run on gel. Samples in PCR reaction (**a)** contains the specified amount of ΔMARR-3 gDNA while samples in second set of PCR reaction (**b)** are normalized to ensure that all the reactions contain a total of 2 µg of DNA. In the absence of DCOA14 gDNA, the empty region was detected using as less as 0.002 ng of gDNA. In the presence of DCOA14 gDNA, the PCR started to detect empty region when 0.02 ng or more gDNA was used as a PCR template (b).

In A424, the Tn*6018*-MARR deleted region was detected when up to 0.2 ng gDNA (roughly 10^4 copies of chromosomes) of was used as a template in the PCR reaction (Figure 5.6). Considering the fact that sensitivity assay can start detecting deletion when 100-1000 copies of target DNA is present in the reaction, it can be inferred that in a population of 10^4 A424 cells, roughly 100-1000 cells harbour the spontaneous deletion of Tn*6018*-MARR. The reactions containing less than 0.2 ng A424 gDNA are not shown in the gel since the visibility of the band was beyond the resolution limit of the agarose gel.

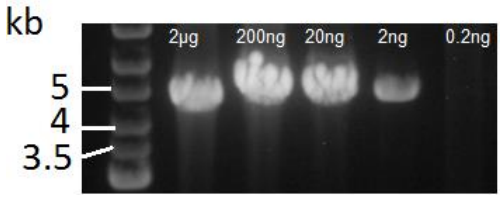

Figure 5.6 Deletion detection test in A424
The detection of deletion within Tn*AbaR23* in A424 using primer pair PR1766 + PR1767. The cycling condition was adjusted to 45 cycles and entire PCR product (20 µl) was run on gel. All the PCR reactions contain specified amount of A424 gDNA and the empty 4.7 kb deleted region was detected using as less as 0.2 ng of gDNA (a very faint band visible on gel).

5.8 Role of *tnpA* in spontaneous deletion of Tn*6018*-MARR

As described in earlier sections, the MARR region in Tn*AbaR23* is bounded between two identical copies of heavy metal transposon Tn*6018*. The central role of Tn*6018*-associated transposase gene *tnpA* in the mobilization of Tn*6018*, previously called IS*Ppu12*, during conjugative interaction was experimentally demonstrated in *Pseudomonas putida* mt-2 (Williams 2002).

In this study, the A424 cells were found to lose Tn*6018*-MARR spontaneously in the absence of antibiotic stress. To check whether Tn*6018*-associated *tnpA* affects spontaneous excision of Tn*6018*-MARR, the two copies of *tnpA* genes in two copies of Tn*6018* within Tn*AbaR23* were targeted for in-frame-insertion-deletion (in-frame-in-del) one at a time. Using the allelic exchange protocol as described in Kochar *et al.* (2012), the pJTOOL-3 based plasmids pKR750 and pKR751 were constructed and employed for the targeted deletion of *tnpA*.

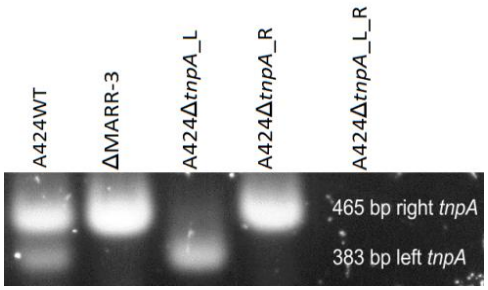

Figure 5.7 Deletion of *tnpA* from left and right Tn*6018* in A424
PCR detection of the presence of left and right *tnpA* in the *tnpA* knockout mutants. Primer pair PR2521 and PR3613 was used to detect the presence of left copy of *tnpA* and primer pair PR3613 and PR3614 was used to detect the presence of right copy of *tnpA*. The presence of double bands in A424 wild type indicates the presence of both copies of *tnpA*; the Tn*6018*-MARR deleted mutant ΔMARR-3 shows the presence of only one copy of *tnpA,* the presence of different sized band in A424Δ*tnpA*_L and A424Δ*tnpA*_R confirms the targeted deletion of left and right copy of *tnpA*. No amplification obtained for the double *tnpA* knock-out mutant A424Δ*tnpA*_L_R as expected indicating the deletion of both the copies of *tnpA*.

Three *tnpA* deleted mutants were created from A424 wild type, the mutant with left copy of *tnpA* deleted is referred here as A424Δ*tnpA*_L, the mutant with right *tnpA* deleted is referred as A424Δ*tnpA*_R and the double knock-out mutant with left and right *tnpA* deleted is referred here as A424Δ*tnpA*_L_R.

The *tnpA* knockout A424 mutants were verified for the loss of their respective transposase gene (Figure 5.7) and were subsequently assessed by semi-quantitative PCR, as described above, to determine the proportion of cells undergoing spontaneous deletion of Tn*6018*-MARR. As described in earlier sections, the excised Tn*6018*-MARR carries with it an intact copy of Tn*6018* including the left and right inverted repeat sequences of Tn*6018*. The primer pair therefore can detect the characteristic 3.7 kb junction (Figure 5.8a) of the circular element formed post excision.

The deletion of *tnpA* would reduce the size of 3.3 kb Tn*6018* to 2.1 kb (Figure 5.8b) and either intact Tn*6018* or Tn*6018*Δ*tnpA* could theoretically reside in the circular element that would form post excision. The primer pair PR2520 and PR2421 can therefore, theoretically, produce either 3.7 kb junction from Tn*6018*-MARR or 2.5 kb junction from Tn*6018*Δ*tnpA*-MARR. When both copies of *tnpA* are deleted, the circular element formed post spontaneous excision would carry Tn*6018*Δ*tnpA* leaving another copy of Tn*6018*Δ*tnpA in situ* and the above primer pair would produce 2.5 kb junction from a circular Tn*6018*Δ*tnpA*-MARR element (Figure 5.8c).

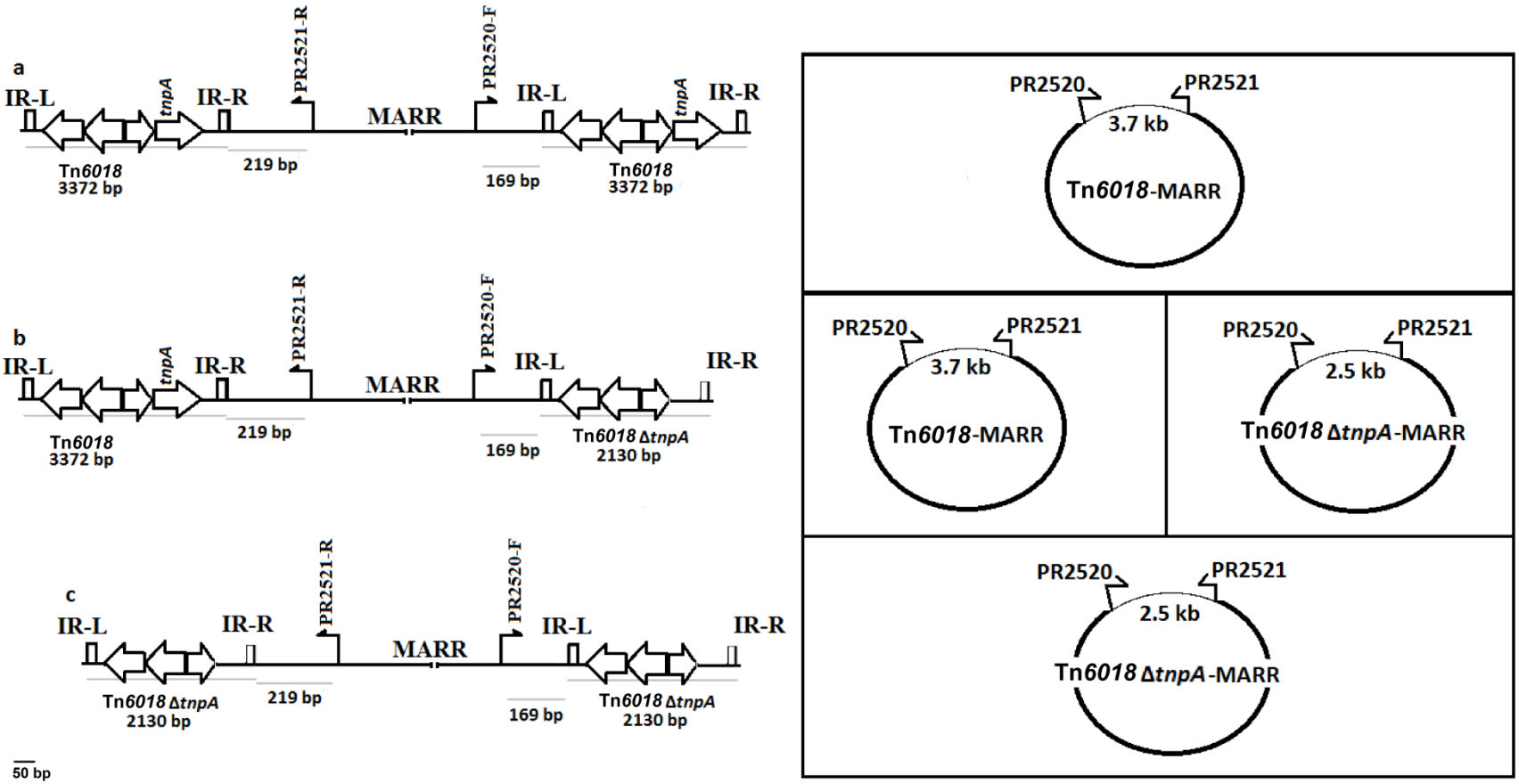

Figure 5.8 Schematics of various circular elements and the calculated size of circular junction

Schematics of Tn*6018*-MARR-Tn*6018* region showing intact transposase gene *tnpA* in both copies of Tn*6018* as in A424 wild type (a), the deletion of *tnpA* from one copy of Tn*6018* (b) and from both copies of Tn*6018* (c). The circular elements and calculated size of circular junction that can be detected by primer pair PR2520 and PR2521 is shown for each mutant. The MARR region being very large is not drawn to scale. The IR-L and IR-R represent the left and right inverted repeat sequences of Tn*6018*. Various sizes of circular junction carrying intact Tn*6018* and truncated Tn*6018*Δ*tnpA* is shown in the box at right.

5.9 Determining the proportion of *tnpA* knock-out mutants exhibiting spontaneous deletion of MARR

A semi-quantitative PCR described as above was carried out to determine the proportion of A424 mutants displaying spontaneous deletion of MARR upon the deletion of left copy, right copy or both copies of *tnpA*.

In a population of 10^4 A424 wild type cells, it was found that around 100 - 1000 cells, more than half of the population, were undergoing the spontaneous deletion of Tn*6018*-MARR. Upon the deletion of either left or right copy of *tnpA,* the deletion detection assay started detecting the presence of empty region when 10^7 or more chromosomes were present in the reaction (Figure 5.9 a, b).

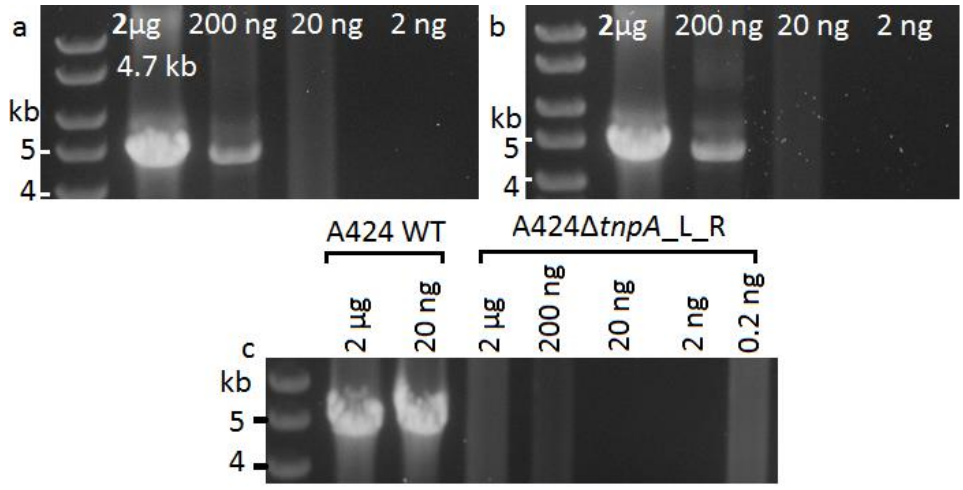

Figure 5.9 Deletion detection assay in various *tnpA* deleted mutants
The cycling condition was adjusted to 45 cycles and entire PCR product (20 µl) was run on gel. The Tn*6018*-MARR deletion in A424Δ*tnpA*_L (a) and A424Δ*tnpA*_R (b) was detected when up to 200 ng of gDNA was used as a PCR template. PCR failed to amplify the deleted region from the double *tnpA* knock-out mutant A424Δ*tnpA*_L_R even when up to 2 µg of gDNA was used as a PCR template (c). In reaction c, A424 gDNA was used as control to show that the assay was functional.

It can therefore be inferred that in a population of 10^7 single *tnpA* knock-out mutant cells, around 100 - 1000 cells exhibited Tn*6018*-MARR deletion which, a 1000 folds decrease compared to A424 wild type carrying two intact copies of *tnpA*. When both the copies of *tnpA* were deleted in the mutant A424Δ*tnpA*_L_R, a visible PCR product

was not detected on the agarose gel even from the reactions where 2 µg of gDNA was used as a template implying that out of 10^8 cells, less than 100 cells could be undergoing the spontaneous deletion of Tn*6018*-MARR.

5.10 Determining the proportion of *tnpA* knock-out mutant cells carrying circular Tn*6018*-MARR element

Upon the deletion of one copy of *tnpA* from Tn*6018*, the primer pair PR2520 and PR2521 could, theoretically, detect two different Tn*6018*-MARR circular element junctions depending on whether an intact Tn*6018* or Tn*6018*Δ*tnpA* is inherited by the circular element (above Figure 5.8). To observe the effect of *tnpA* deletion in formation of circular Tn*6018*-MARR element, a PCR based semi-quantitative assay described as above was used to determine the proportion of cells carrying Tn*6018*-MARR circular element in the tnpA knock-out mutants created during this study.

In circular junction detection sensitivity assay, out of around 10^5 A424 gDNA, the circular junction was detected in around 100 – 1000 cells. In the single and double *tnpA* knock-out mutants, a visible PCR amplicon from the Tn*6018*-MARR circular junction was not detected on the agarose gel when up to 2 µg of gDNA was as template in the PCR reaction (Figure 5.10). This could mean that although from a population of 10^7 single *tnpA* knock-out mutants 100-1000 cells were undergoing spontaneous deletion of Tn*6018*-MARR, the circular element appeared to be forming in a much smaller sub-population (less than 100-100 cells in a population of 10^8 cells).

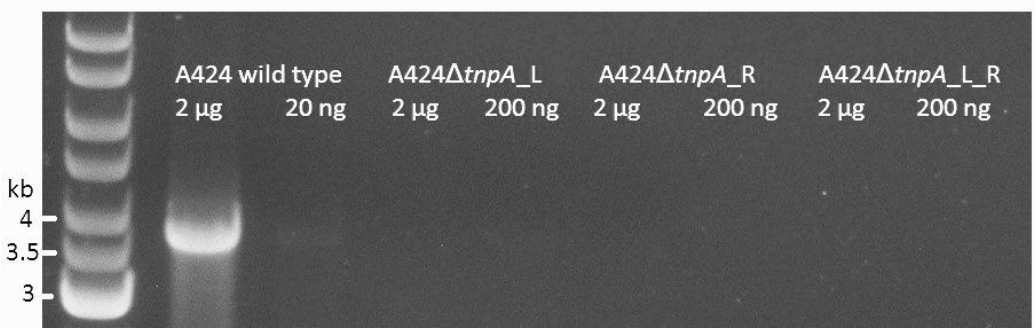

Figure 5.10 Circular junction detection assay
The junction of Tn*6018*-MARR circular element in A424Δ*tnpA*_L, A424Δ*tnpA*_R and A424Δ*tnpA*_L_R mutants was detected using the primer PR2520 and PR2521. The cycling condition was adjusted to 45 cycles and entire PCR product (20 µl) was run on gel. Indicated amount of gDNA was used in each reaction tube. A424 wild type gDNA was used as a control to show that the assay was functional. Faint band observed for A424 WT 20 ng gDNA.

5.11 Discussion and conclusion

5.11.1 Plasticity of AbaR islands

The AbaR elements in *A. baumannii* are considered to be acquired from various bacteria like *Pseudomonas* spp., *E. coli* and *Salmonella* spp. by means of horizontal gene transfer (Fournier *et al.* 2006). These elements subsequently evolved and diversified during multiple events of insertion, deletion and recombination mostly mediated by insertion sequence elements and transposons (Krizova *et al.* 2011). The AbaR islands seem to be stably integrated in the chromosome of their host and spontaneous deletion or transposition of the intact islands has not been reported yet in any *A. baumannii* strains. In this study, for the first time, the spontaneous deletion of a large region internal to AbaR1 and Tn*AbaR23* in MDR *A. baumannii* strains AYE and A424 is reported. In this study, a sub-population of AYE and A424 were found to spontaneously lose Tn*6018*-MARR region from their AbaR islands under the laboratory *in vitro* growth conditions and in the absence of antibiotic stress. The spontaneously excised double stranded (ds) Tn*6018*-MARR element was found to attain a circular form and was subsequently lost in its entirety from the cell giving rise to a sub-population of A424 carrying truncated Tn*AbaR23*. Although the circular Tn*6018*-MARR

was not visualized or physically detected, its presence in a sub-population of A424 cells is inferred from the circular element junction PCR amplicon.

A total of four independent Tn*6018*-MARR deleted spontaneously arising mutants were isolated from a population of A424 wild type by parallel-plate patching based on the susceptibility of spontaneous mutants towards sulphametoxazole 40 µg/ml. The Tn*6018*-MARR deleted strain screening was executed using a protocol that ensured no prior exposure of the selected Tn*6018*-MARR deleted mutants to antibiotics. The deletion of Tn*6018*-MARR in spontaneous mutants was verified by PCR. The PFGE band pattern of all four spontaneous mutants appeared indistinguishable and the PFGE profile was consistent with the deletion of Tn*6018*-MARR.

In each of the four Tn*6018*-MARR deleted mutants studied, the MARR was inferred being excised as a double stranded circular structure that carried a single copy of Tn*6018* transposon, with the second copy of this element remaining *in situ* within the now markedly truncated Tn*AbaR23*. Importantly, PCR analysis verified that following excision from its native site that the MARR segment did not re-integrate elsewhere in the genomes of the four Tn*6018*-MARR deleted mutants investigated, but that it was lost in entirety from these strains.

In a population of 10^4 A424 wild type cells, around 100 - 1000 cells were found to be losing Tn*6018*-MARR spontaneously as detected by the semi-quantitative PCR assay. However, the circular Tn*6018*-MARR entity was possessed by around 100 -1000 cells in a population of 10^5 A424 wild type cells as detected by the semi-quantitative PCR assay. The sub-population of A424 cells carrying circular Tn*6018*-MARR appears to be 10 folds less that the sub-population undergoing spontaneous deletion of Tn*6018*-MARR. It is possible that, post excision, a small proportion of Tn*6018*-MARR reintegrated at their native site instead of forming a circular element. A very high proportion of A424 cells (around 4 in 1200) were found to lose Tn*6018*-MARR from their Tn*AbaR23* as determined by the colony patching method. However, caution must be applied before accepting that 4 in 1200 A424 cells were spontaneously losing

Tn*6018*-MARR since a small population of A424 cells were screened for their sulfamethoxazole sensitivity.

As discussed earlier, the spontaneous deletion of Tn*6018*-MARR was also observed in AbaR1 island from MDR strain AYE. Given the fact that large proportion of A424 cells exhibited spontaneous deletion of Tn*6018*-MARR to give rise to a sub-population of sulfamethoxazole sensitive mutants carrying truncated Tn*AbaR23*, it can be hypothesized that the spontaneous deletion and loss of Tn*6018*-MARR observed in this study could be a common phenomenon that might be very frequently happening in several AbaR islands, at least in *in vitro* growth conditions.

5.11.2 Role of *tnpA* in spontaneous deletion of Tn*6018*-MARR from Tn*AbaR23*

In the strain A424, the *tnpA* from left and right Tn*6018* were deleted individually creating a left *tnpA* knock-out mutant A424Δ*tnpA*_L, a right *tnpA* knock-out mutant A424Δ*tnpA*_R and a double *tnpA* knock-out mutant A424Δ*tnpA*_L_R. A 1000 fold decrease in the rate of spontaneous deletion of Tn*6018*-MARR was observed when *tnpA* was deleted from either left or right copy of Tn*6018* as evident by the semi-quantitative PCR where upon the deletion of only one copy of *tnpA*, 100 – 1000 cells in every 10^7 cells were found to undergo a spontaneous deletion of Tn*6018*-MARR region. Thus, the pre-requisite for the high rate of spontaneous deletion of this nature appears to be the presence of at least one copy of *tnpA* gene in either of the two copies of Tn*6018* from the compound transposon Tn*6018*-MARR-Tn*6018.* It is also likely that one copy of *tnpA* could provide a threshold amount of Transposase to drive the spontaneous deletion of Tn*6018*-MARR as observed in A424Δ*tnpA*_L and A424Δ*tnpA*_R mutants. When *tnpA* was deleted from both copies of Tn*6018* in A424, the semi-quantitative PCR assay failed to detect the spontaneous deletion of Tn*6018*-MARR. Intriguingly, it appears that for the excised Tn*6018*-MARR element to exist as a circular entity, presence of intact copies of two *tnpA* gene appears to be essential.

Theoretically, the excised Tn*6018*-MARR could form two different sizes of ds circular Tn*6018*-MARR in single *tnpA* knock-out mutants. Remarkably, the junction of Tn*6018*-

MARR circular element was not detected in all three *tnpA* deleted mutants. Failure to detect the circular junction A424Δ*tnpA*_L and A424Δ*tnpA*_R shows that despite the occurrence of spontaneous deletion within Tn*AbaR23*, the circular Tn*6018*-MARR element could not form in these single *tnpA* knock-out mutants. It is likely that in the absence of two copies of functional *tnpA*, the Tn*6018*-MARR failed to attain a circular state post excision and re-integrated into its native site. Equally, it is possible that the region available for the homologous recombination were shorter in single *tnpA* and double *tnpA* knock-out mutants which results in the reduction of spontaneous excision of Tn*6018*-MARR. This could explain why the spontaneous deletion was detected but the circular element was not detected in single *tnpA* knockout mutants. It is also likely that two copies of intact *tnpA* were prerequisite for the Tn*6018*-MARR to exist as a circular entity. The presence of Tn*6018*-MARR as a circular entity was inferred on the basis of detection of circular element junction by PCR and, in this instance, there is no physical evidence of the presence of different sized Tn*6018*-MARR circular element in *tnpA* knock-out mutants.

Study on the movement of the antibiotic resistance genes flanked by two directly oriented copes of IS*26* (Harmer *et al.* 2014), in a *recA* negative strain of *E. coli* showed that one copy of IS*26* together with the antibiotic resistance genes forms a mobile transposable unit (the authors called it "translocatable unit"). The authors went on explaining that this translocatable unit recognizes IS*26* as a target and a high frequency of recombination between translocatable units at its target occurs when both of the IS*26* possess active transposase Tnp26. During this study, the exact mechanism of Tn*6018*-MARR excision, its target site selection and its mode of transposition (if there is any) could not be investigated due to time constraints but it is possible that Tn*6018*-MARR entity can behaves like the IS*26*-like translocatable unit in-terms of target site selection and mode of transposition. In this study, a 1000 fold decrease in spontaneous excision of Tn*6018*-MARR was observed when one copy of Tn*6018* transposase *tnpA* was knocked-out and, intriguingly, the spontaneous excision was not detected when both the copies of *tnpA* were knocked-out. In consistent with Harmer *et al.* (2014) findings, the importance of active transposase for the Tn*6018*-MARR excision has been clearly demonstrated in this study. Importantly, the circularization of Tn*6018*-MARR

entity was not detected by PCR despite lower rate of Tn*6018*-MARR excision in mutants with one copy of *tnpA* knocked-out. It is likely that the length of region available for homologous recombination is also important for the high rate of excision and circularization of Tn*6018*-MARR and that a functional Tn*6018* might not be required for the excision of Tn*6018*-MARR.

This study demonstrates the precise deletion and circularization of Tn*6018*-MARR from Tn*AbaR23* in strain A424. The circularized Tn*6018*-MARR was not obtained as a physical entity in this study but it would be fascinating to study its transposition ability and efficiency in order to better understand the contribution of these elements in the development of traits and characteristics that are vital for the survival of the strain in the environment. In the next chapter, the Tn*6018*-MARR deleted spontaneous mutants will be further analysed for their contribution on the phenotype of A424 strain.

6 Contribution of Tn*AbaR23* island on the phenotype of *Acinetobacter baumannii* strain A424

6.1 Background

In the introduction chapter, I have briefly described the study conducted by Kochar *et al.* (2012) and their conclusions regarding the contribution of Tn*AbaR23* on the antibiotic resistance phenotype of A424. The author reported that despite having the wild type *gyrA* and *parC* genes the Tn*AbaR23* deleted mutant DCO174 was found to show an enhanced resistance to ciprofloxacin with MIC 8 fold higher than its parent A424. Importantly, the author hypothesized that the unusual ciprofloxacin resistance exhibited by DCO174 could be due to either the deletion of Tn*AbaR23* or a cryptic secondary mutation in the efflux gene.

Importantly, AbaR islands were considered to contribute minimally towards the overall antibiotic resistance phenotype of the host strain (Krizova *et al.* 2011). To validate this claim, the published literatures were searched to compile data (Table 6.1) on the antibiotic resistance conferring genes and determinants on AbaR island and the antibiotic resistance phenotype of the associated host strain.

Table 6.1 Antimicrobial susceptibility profile of MDR *A. baumannii* strains

Strain, AbaR island, tested drug susceptibility phenotype*		**AbaR associated genes and determinants for antibiotic and heavy metal resistance**	**References**
WM98 **AbaR0 MDR**	Resistant to gentamicin, ciprofloxacin, cefotaxime, ticarcillin, timentin, ampicillin, cephalothin, aztreonam′	sulphonamide, tetracycline, neomycin, spectinomycin, streptomycin, kanamycin, arsenic, antimony, chloramphenicol, cadmium, mercury, quaternary ammonium compounds	(Valenzuela *et al.* 2007, Hamidian *et al.* 2014b)
AYE **AbaR1 MDR**	Resistant to tetracycline, carbenicillin, ampicillin, ticarcillin-clavulanate, cefotaxime, ciprofloxacin, gentamicin, trimethoprim, rifampacin, chloramphenicol, sulfamethoxazole and sulfamethoxazole-trimethoprim. Susceptible to imipenem	sulphonamide, tetracycline, aminoglycosides, streptomycin, spectinomycin, neomycin, kanamycin, arsenic and antimony, chloramphenicol, cadmium, mercury quaternary ammonium compounds, β-lactams	(Fournier *et al.* 2006, Vallenet *et al.* 2008, Kochar *et al.* 2012)
ACICU AbaR2 MDR	Resistant to fluoroquinolones, carbapenems, piperacillin, piperacillin-tazobactam, ceftazidime, ciprofloxacin, chloramphenicol, trimethoprim, sulfamethoxazole, tobramycin Susceptible to gentamicin, colistin, sulbactam,	Sulphonamide, aminoglycoside, truncated gene for kanamycin and neomycin resistance, quaternary ammonium compounds	(Longo *et al.* 2006, Iacono *et al.* 2008)
A85 **AbaR3** **And** **AbaR4** **XDR**	Resistant to rifampicin, ciprofloxacin; cefotaxime, ceftazidime, ampicillin/sulbactam, imipenem, meropenem, ticarcillin, clavulanic acid, spectinomycin, tetracycline, trimethoprim, kanamycin, gentamicin, ticarcillin/clavulanate, sulbactam fluoroquinolones, nalidixic acid, ciprofloxacin	Sulphonamide, tetracycline, gentamicin, streptomycin, spectinomycin, , kanamycin, neomycin, , ticarcillin/clavulanate, imipenem, meropenem, β-lactams	(Kenyon and Hall 2013)
3208 AbaR5 MDR	resistant to gentamicin, ciprofloxacin, trimethoprim, and sulfamethoxazole intermediate resistance to ceftriaxone, ceftazidime, cefepime and ticarcillin-clavulanate susceptible to piperacillin-tazobactam, tobramycin and meropenem	Sulphonamide, tetracycline, gentamicin, arsenic and antimony, streptomycin, spectinomycin, cadmium, mercury kanamycin, neomycin	(Post and Hall 2009)
D2 **AbaR6**	Resistant to cefalexin, cefazolin, chloramphenicol, sulfafurazole, nalidixic acid, tigecycline, streptomycin,	Sulphonamide, gentamicin, streptomycin, spectinomycin cadmium, kanamycin, neomycin,	(Mak *et al.* 2009, Post *et*

Strain, AbaR island, tested drug susceptibility phenotype*		AbaR associated genes and determinants for antibiotic and heavy metal resistance	References
MDR	kanamycin, neomycin, gentamicin	quaternary ammonium compounds	*al.* 2010)
A424 Tn*AbaR23* MDR	Resistant to trimethoprim, sulfamethoxazole, sulfamethoxazole-trimethoprim, chloramphenicol, tetracycline, carbenicillin, ampicillin, ticarcillin-clavulanate, cefotaxime, ciprofloxacin, gentamicin, rifampacin, Susceptible to gentamicin and imipenem	sulphonamide, tetracycline, gentamicin, arsenic, antimony streptomycin, spectinomycin, chloramphenicol, , cadmium, mercury	(Kochar *et al.* 2012)
BJAB07104AbaR25 MDR	Resistant to amikacin, caftazidime, cefepime, cefotaxime, cefoperazone, ciprofloxacin, imipenem, levofloxacin, meropenem, minocycline, piperacillin, piperacillin/tazobactam, tetracycline, sulbactam, meropenem Susceptible to polymixin	sulphonamide, tetracycline , aminoglycoside arsenic, antimony, β-lactams	(Zhu *et al.* 2013)
BJAB0868 AbaR26 MDR	Resistant to amikacin, caftazidime, cefepime, cefotaxime, ciprofloxacin, imipenem, levofloxacin, meropenem, minocycline, piperacillin, tazobactam, polymixin, tetracycline Susceptible to polymixin and minocycline	sulphonamide, tetracycline, aminoglycoside, arsenic, antimony, β-lactams	(Zhu *et al.* 2013)
BJAB0715 AbaR27 MDR	Resistant to amikacin, caftazidime, cefepime, cefotaxime, ciprofloxacin, imipenem, levofloxacin, meropenem, minocycline, piperacillin, tazobactam, polymixin, tetracycline Susceptible to polymixin	sulphonamide, tetracycline, aminoglycosides, arsenic, antimony	Zhu, Yan *et al.* 2013)

*The antimicrobial resistance phenotype of *A. baumannii* strains was obtained from the published literatures.

The maintenance and preservation of AbaR islands solely aiming to enhance the resistance towards few antimicrobial agents that are not currently used in treatment seems to be an inadequate explanation given the fact that AbaR associated resistance determinants played a minimum role towards the overall multi-drug-resistance phenotype of the host strain (Table 6.1). Therefore it is possible that along with drug resistance phenotypes, AbaR islands in MDR *A. baumannii* strains are associated with other traits that are vital for the propagation of *A. baumannii*.

In this chapter, I am intending to investigate the phenotypes associated with Tn*AbaR23* in strain A424. Both of the Tn*AbaR23* deleted mutants DCO163 and DCO174 reported by Kochar *et al.* (2012) were unsuitable to use in this phenotypic comparison study due to their unintended genomic rearrangements (DCO163) and unexpected resistance phenotype (DCO174). Therefore I will be creating new independent Tn*AbaR23* deletion mutants with expected genotype and compare the fitness and virulence phenotypes of A424 wild type and Tn*AbaR23* deleted mutants using various *in vitro* and *in vivo* assays.

Here, I will also investigate the association of Tn*AbaR23* deletion on enhanced ciprofloxacin resistance in new independent Tn*AbaR23* deleted mutants. The comparative sequence analysis of A424 and DCO174 (Crosatti *et al.* unpublished data 2013) revealed a substitution of nucleotide G to A at 2601753 position on the chromosome of DCO174. This single nucleotide polymorphism (SNP) was reported to lie within the *adeS* gene, regulator of AdeABC efflux pump, and it appeared to be solely responsible for the elevated ciprofloxacin resistance in DCO174 (Crosatti *et al.* unpublished data 2013). In this study, the impact of this *adeS*-associated SNP (here referred as $adeS^{G178A}$) on the ciprofloxacin resistance of the A424 strain will be investigated. Experiments will be designed to swap the *ades* allele (here referred as $adeS^{WT}$) present in various A424 strain background with mutated $adeS^{G178A}$. The phenotypes of the strains carrying mutated $adeS^{G178A}$ and wild type $adeS^{WT}$ will be subsequently compared in various *in vitro* assays.

6.2 Aims and objectives

Experiments were designed to create the Tn*AbaR23* deleted mutants for phenotypic comparison with parent A424 wild type. The phenotypes of Tn*6018*-MARR deleted spontaneous mutants lacking Tn*6018*-MARR (reported in chapter 4) was also studied in various *in vivo* and *in vitro* assays. The experiments were designed and conducted in order to-

- delete Tn*AbaR23* island *en bloc* and create markerless mutant
- complement the Tn*AbaR23* deleted mutants with 4 – 6 kb blocks of Tn*6018*-MARR fragments
- assess the antibiotic resistance profile of A424 wild type and its various mutants
- compare the fitness of A424 wild type and its various mutants in an *in vitro* head-to-head competition assay
- compare the virulence of wild type and its various mutants in *Galleria mellonella* killing assay
- replace the $adeS^{WT}$ with $adeS^{G178A}$ in various A424 strain background
- assess the antibiotic resistance profile of the strains carrying mutant $adeS^{G178A}$

6.3 Results

6.3.1 Modified protocol for *en bloc* deletion of Tn*AbaR23*

The protocol and the vector pJTAG used for the deletion of the *TnAbaR23* were previously described by Kochar *et al.* (2012). In brief, the pJTOOL-3 derived deletion vector pJTAG (Figure 6.1) carrying gentamicin resistance marker *aacC1* was conjugally delivered into recipient *A. baumannii* strain A424 in an overnight conjugating using S17.1λpir_pJTAG as a donor strain. Post conjugation, the transconjugants were plated on Simmon's citrate agar (SCA) supplemented with various concentrations of gentamicin aiming to select the SCO mutants with the lowest possible amount of antibiotic exposure.

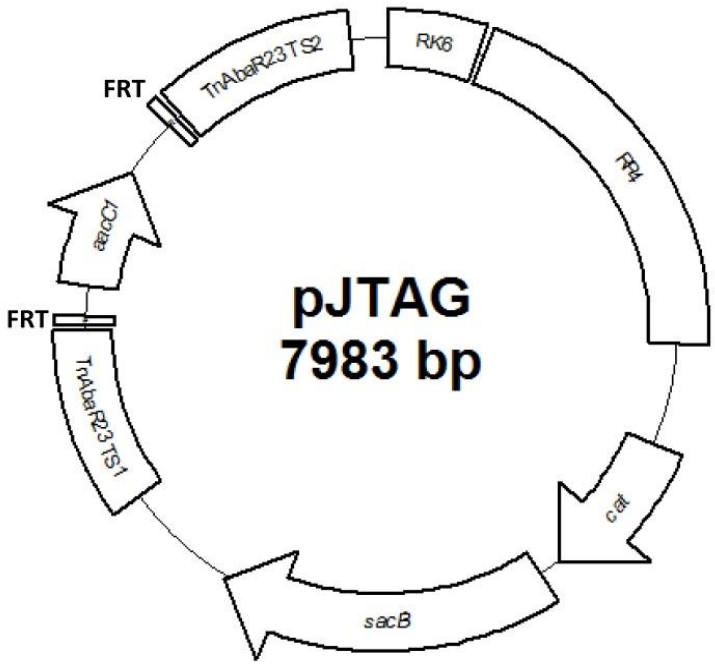

Figure 6.1 Schematics of suicide vector pJTAG
Key genes include chloramphenicol acetyltransferase *cat,* levansucrase *sacB* and aminoglycoside 3'-acetyl transferase *aacC1*. The plasmid contains origin of replication RK6, feature RP4. The flipase recognition target sites (FRT) flanked *aacC1* cassette conferring gentamicin resistance is bracketed by the targeting sequences Tn*AbaR23* TS1 and Tn*AbaR23* TS2 from the AbaR island in strain A424. Plasmid was drawn using sequence information from Kochar *et al.* (2012).

The authors reported the use of 100 µg/ml gentamicin in the media to select and maintain the single cross over (SCO) and DCO mutants. In this study, the SCO and DCO

mutants were selected and maintained ensuring the lowest possible dose of antibiotic exposure as detailed in Table 6.2. A424WT recipient and S17.1λpir_pJTAG donor were mixed in 1:1 ratio for overnight conjugation on agar plate. A 100 µl aliquot of conjugation suspension at various dilutions was plated on SCA supplemented with various concentrations of gentamicin to select for the single cross over A424 mutants. A424 WT was set as a recipient conjugation control and S17.1λpir was set as donor conjugation control.

From two independent conjugation experiments described above, putative transconjugants growing on SCA supplemented with 6.25 µg/ml gentamicin were randomly picked and PCR verified for the incorporation of pJTAG either left or right of Tn*AbaR23*. The independent right SCO5 (pJTAG incorporated at the right of Tn*AbaR23*) and left SCO1 (pJTAG incorporated at the left of Tn*AbaR23*) were archived in the lab collection as KR3178 and KR3205. Unless otherwise stated, the SCO mutants were grown and maintained on media containing 6.25 µg/ml gentamicin.

Table 6.2 Conjugative delivery of pJTAG into A424 and selection of SCO mutants

Culture	**Dilution plated**	**SCA supplemented with gentamicin (concentration μg/ml)**								
		100	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.56**	**0.78**	**0.39**
Mixed culture A*	Undiluted	0	2	haze	35	228	Lawn	Lawn	Lawn	Lawn
Mixed culture A	10^{-1}	0	0	1	1	2	Lawn	Lawn	Lawn	Lawn
Mixed culture A	10^{-2}	2	0	0	1	0	haze	Lawn	Lawn	Lawn
Mixed culture A	10^{-3}	0	0	0	0	0	haze	Lawn	Lawn	Lawn
Mixed culture B**	Undiluted	0	3	0	22	121	Lawn	Lawn	Lawn	Lawn
Mixed culture B	10^{-1}	0	0	0	haze	21	Lawn	Lawn	Lawn	Lawn
Mixed culture B	10^{-2}	2	0	0	haze	0	haze	Lawn	Lawn	Lawn
Mixed culture B	10^{-3}	0	0	0	haze	0	haze	Lawn	Lawn	Lawn
A424 WT	Undiluted	0	0	0	0	0	Lawn	Lawn	Lawn	Lawn
A424 WT	10^{-1}	0	0	0	0	0	haze	Lawn	Lawn	Lawn
A424 WT	10^{-2}	0	0	0	0	0	haze	Lawn	Lawn	Lawn
A424 WT	10^{-3}	0	0	0	0	0	haze	Lawn	Lawn	Lawn
S17λ*pir* _ pJTAG	Undiluted	0	0	0	0	0	0	ns	ns	Lawn
S17λ*pir* _ pJTAG	10^{-1}	0	0	0	0	0	0	ns	ns	Lawn
S17λ*pir* _ pJTAG	10^{-2}	0	0	0	0	0	0	ns	ns	Lawn
S17λ*pir* _ pJTAG	10^{-3}	0	0	0	0	0	0	ns	ns	Lawn

*Mixed culture A is first independent set of the conjugation and **mixed culture B is the second independent set of the conjugation. ns = numerous pin prick sized colonies

6.3.2 Creation of new independent Tn*AbaR23* deleted mutants

The independent SCO mutants KR3178 and KR3205 were used to create independent Tn*AbaR23* deleted mutants during sucrose counter-selection. In brief, a suspension of SCO mutants grown on antibiotic free media were plated on LA plate supplemented with 6 % sucrose. The suicide vector pJTAG carries on its backbone a sucrase gene called *sacB*. In the presence of sucrose, an intermediate product called levansucrase is formed which is toxic to the cells. In order to survive in the media containing sucrose, the single cross-over mutant must get rid of pJTAG integrated on its chromosome. This provokes a second homologous recombination event between the two copies of Tn*AbaR23* targeting sequence 1 [TS1] or Tn*AbaR23* targeting sequence 2 [TS2].

During the second homologous recombination event, the plasmid pJTAG would be lost from the chromosome and depending on where the homologous recombination has occurred [TS1 or TS2], the colonies growing on sucrose plate could be either the mutants reverted to wild type [only pJTAG lost from chromosome] or the Tn*AbaR23* island deleted mutants [pJTAG and Tn*AbaR23* lost from the cell]. The sucrose counter-selection was carried out using two independent SCO mutants. A 1000 fold decrease in the number of colonies on sucrose plates was observed during counter-selection (Table 6.3). The gentamicin resistant putative DCO mutants were validated for the loss of Tn*AbaR23*. Independent TnAbaR23 deleted mutants were archived in the laboratory strain collection.

Table 6.3 Sucrose counter-selection for creation of DCO mutant

Strain	Dilution	LA		LA + 6% sucrose media		Parallel patching	
		CFU	CFU/ml	CFU	CFU/ml	LA	LA + gentamicin
SCO5	10^{-1}	np	3.5 - 3.8 × 10^7	110	0.7 - 1.1 × 10^4	25	5
	10^{-2}	np		7			
	10^{-3}	np		0			
	10^{-4}	387		np			
	10^{-5}	35		np			
SCO1	10^{-1}	np	2.8 - 3.0 × 10^7	20	0.5 - 2.0 × 10^4	25	10
	10^{-2}	np		5			
	10^{-3}	np		0			
	10^{-4}	301		np			
	10^{-5}	28		np			

The SCO5 and SCO1 are independent single cross over mutants created in this study and have pJTAG plasmid integrated in their chromosomes. A 100 µl aliquot of bacterial suspension at various dilutions was plated to count the CFU. A total of 25 colonies growing on sucrose media were parallel patched on LA containing 6.25 µg/ml gentamicin to confirm the gentamicin resistance upon the acquisition of resistance marker. The colonies growing on gentamicin plate were verified for the loss of Tn*AbaR23* island and deletion suicide vector pJTAG. **= np plated

6.3.3 Verification of new independent Tn*AbaR23* deleted mutants

Post sucrose counter-selection, putative DCO mutant colonies on 6 % sucrose were parallel patched on LA and on LA supplemented with 6.25 µg/ml gentamicin to confirm their gentamicin resistance (Table 6.3). A total of 10 gentamicin resistant putative DCOs were randomly picked (five derived from each SCOs) and were tested for the deletion of Tn*AbaR23* island, loss of the pJTAG backbone and presence of the marker gene *aacC1*. From two independent SCOs, a total of four putative DCOs C2, C3, C4 and C5 were found negative for the presence of Tn*AbaR23* island gene *tniA* indicating the possible deletion of island (Figure 6.2a). Genomic DNA (gDNA) was extracted from C2, C3, C4 and C5 to detect empty *comM* using primers that anneal outside 5' end and 3' end of *comM* gene. The amplification of 3.7 kb product in all tested validated the deletion of Tn*AbaR23* island from *comM* (Figure 6.2b) and the negative *sacB* PCR in all putative DCOs validated the loss of pJTAG plasmid (Figure 6.2c). The independent Tn*AbaR23* deleted DCO mutants derived from independent SCO mutants were archived as KR3075, KR3223, KR3204 and KR3220 in the laboratory collection (see strain table in method section). For mutants DCOA14 (KR3075) and DCOC5-4 (KR3220),

the absence of unintended genetic rearrangements and the presence of targeted deletion of Tn*AbaR23* were validated by PFGE.

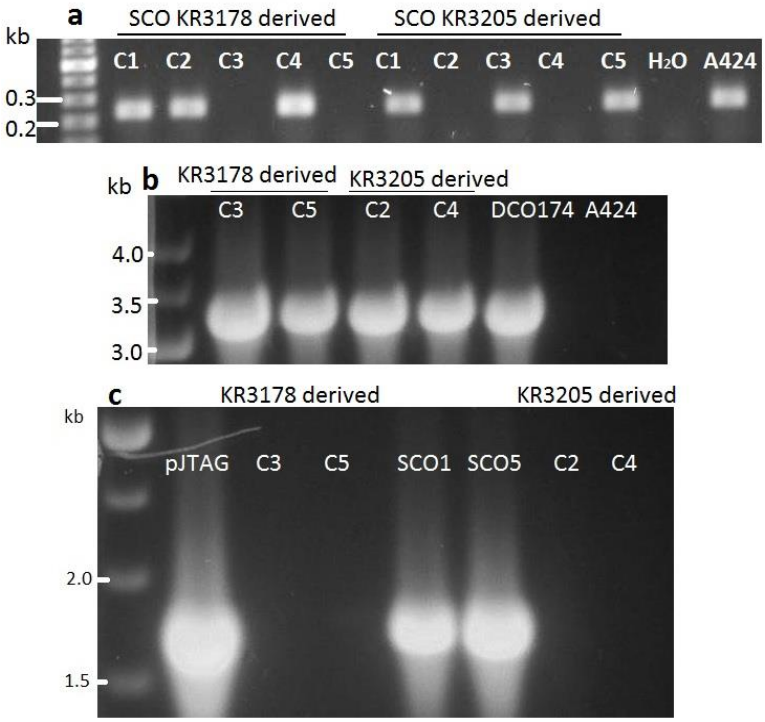

Figure 6.2 Verification of Tn*AbaR23* deleted mutants
Out of five gentamicin resistant putative DCOs, KR3178 derived putative DCOs C3 and C5 and KR3205 derived putative DCOs C2 and C4 showed the absence of Tn*AbaR23* island gene *tniA* indicating a possible loss of Tn*AbaR23* (a). Primer pair annealing outside the Tn*AbaR23* island amplified 3.7 kb product in *tniA* negative putative DCOs C2, C3, C4 and C5 verifying the deletion of Tn*AbaR23* from *comM* (b). The negative PCR for the pJTAG backbone gene *sacB* in DCOs C2, C3, C4 and C5 validates the loss of pJTAG from their cells during sucrose counter-selection (c).

6.3.4 PFGE of new Tn*AbaR23* deleted mutants

The *NotI* digested high molecular weight gDNA of parent A424 wild type and two new independent mutants DCOA14 and DCOC5-4 was subjected to PFGE (Figure 6.3). The band pattern of previously reported DCO174 and two independent mutants DCOA14 and DCOC5-4 created in this study was indistinguishable suggesting that the strains are likely to be isogenic. Also, the band pattern in all the tested Tn*AbaR23* deleted mutants was consistent with the deletion of the island and band discrepancy between two new independent DCO mutants DCOA14 and DCOC5-4 was not observed. The

PFGE band profile did not show any unintended genomic changes or rearrangements in the tested DCO mutants.

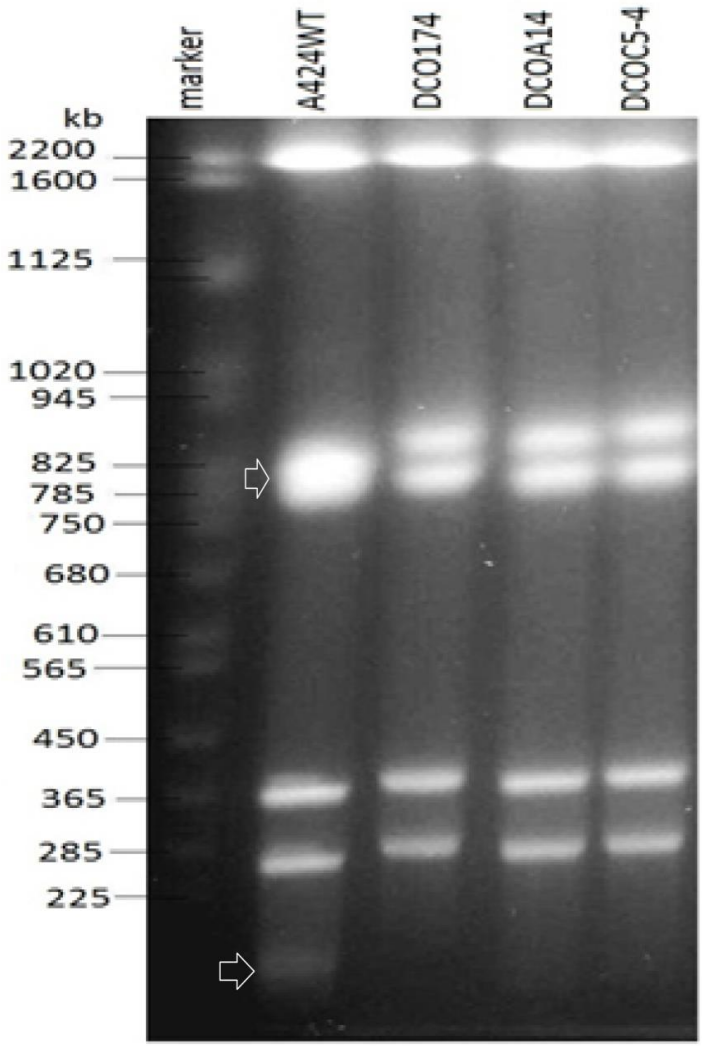

Figure 6.3 Verification of new independent DCOs by PFGE
The *NotI* digested genome of A424WT, DCOA14, DCOC5-4 DCO174 were run alongside *Saccharomyces cereviseae* marker in PFGE. The PFGE profile of the mutant strains was indistinguishable from one another suggesting that the TnAbbR23 deleted strains are likely to be isogenic. The A424 wild type specific bands are indicated with white arrow in the gel image.

6.3.5 Rendering the Tn*AbaR23* deleted DCO mutant markerless

The FRT flanked *aacC1* cassette in DCOA14 was excised in a flipase mediated recombination event using vector pFLP2_*sul1*. In short, the vector pFLP2_*sul1* was constructed by swapping its ampicillin resistance conferring gene *bla* with

sulfamethoxazole resistance conferring gene *sul1* gene in a lambda red homologous recombination event facilitated by pKOBEG_Apra (Figure 6.4).

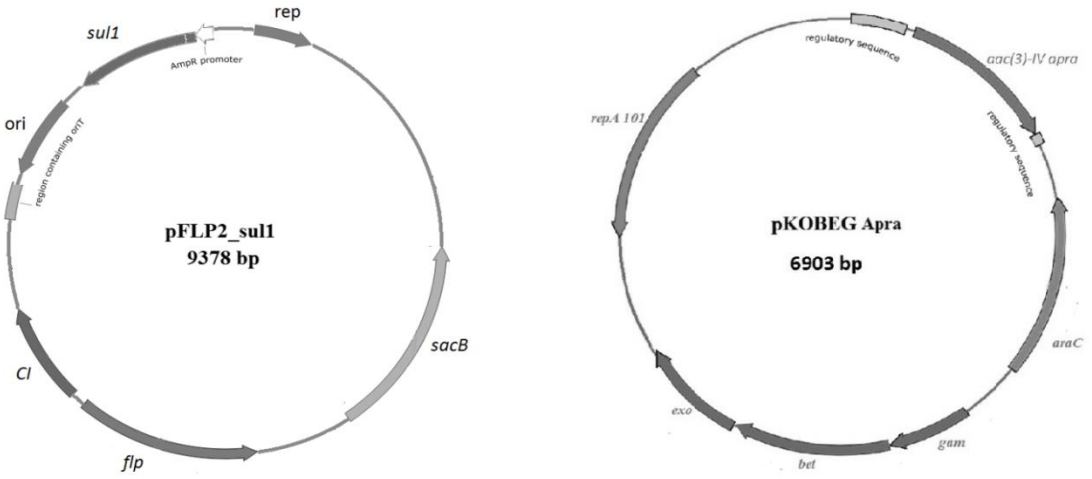

Figure 6.4 Schematics of plasmids pFLP2_*sul1* and pKOBEG_Apra
The plasmid pFLP2 _*sul1* contains key genes sacB that codes for a toxic intermediate product levansucrase. The flipase gene *flp* is under the control of lambda repressor *CI*. The Plasmid pKOBEG Apra contains key red genes *gam, bet* and *exo* which are under the control of arabinose inducible promoter. The gene *araC* codes for arabinose and repA101 is a temperature sensitive region. The gene *aac(3)-IVapra* codes for aminoglycoside-modifying enzyme aminoglycoside 3-N-acetyltransferase type-IV.

The plasmid pFLP2-*sul1* was electroporated into DCOA14 and the transformed cells were recovered in SOC media at 37°C for 1.5 h before plating onto MHA supplemented with 40 µg/ml sulfamethoxazole. From 100 µl of transformed cell suspension, 78 putative transformed colonies were obtained from which five colonies were randomly picked and purified for the verification of loss of *aacC1* cassette. Out of five colonies tested, two colonies (C1 and C2 in Figure 6.5) were validated for the loss of *aacC1* cassette. Markerless Tn*AbaR23* deleted mutants DCOA14-Δ7 and DCOA14-Δ18 were archived in the laboratory strain collection as KR3873 and KR3874.

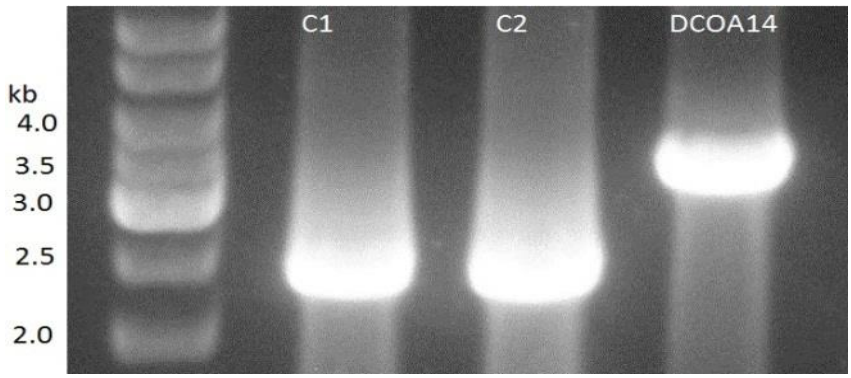

Figure 6.5 Verification of markerless mutants derived from DCOA14
PCR across the *comM* gene from where Tn*AbaR23* has knocked-out and the gentamycin resistance conferring marker *aacC1* has been integrated produced 3.7 kb band in DCOA14 showing the presence of marker. A smaller 2.7 kb band in the markerless mutant colonies C1 and C2 produced by the same primer pair indicated the loss of ~1 kb *aacC1* marker.

6.3.6 Complementation of blocks of Tn*6018*-MARR region in spontaneous mutant ΔMARR-3

As described earlier in chapter 5, the Tn*6018*-MARR segment from Tn*AbaR23* was found to excise and lost spontaneously in a sub-population of A424 cells. A total of four independent spontaneous mutants were isolated and verified for the presence of truncated 19.7 kb Tn*AbaR23* and absence of Tn*6018*-MARR. Out of four spontaneous mutants, ΔMARR-3 (KR3326) was randomly picked as a representative for use in various phenotypic assays. Various blocks of Tn*6018*-MARR region ranging from 3.6 – 6.4 kb were cloned in pWH1266-Gm, an *E. coli - A. baumannii* shuttle vector, for the plasmid complementation of Tn*6018*-MARR fragments in the spontaneous mutant ΔMARR-3 (Figure 6.6). A total of 7 complementation plasmids were created each carrying one block of Tn*6018*-MARR fragment. The plasmids were then electroporated into the ΔMARR-3 mutant and transformed cells were selected on 6.25 µg/ml gentamicin. The stability of complementation plasmids in ΔMARR-3 was confirmed by the recovery of plasmids and verification by restriction digestion (Figure 6.6).

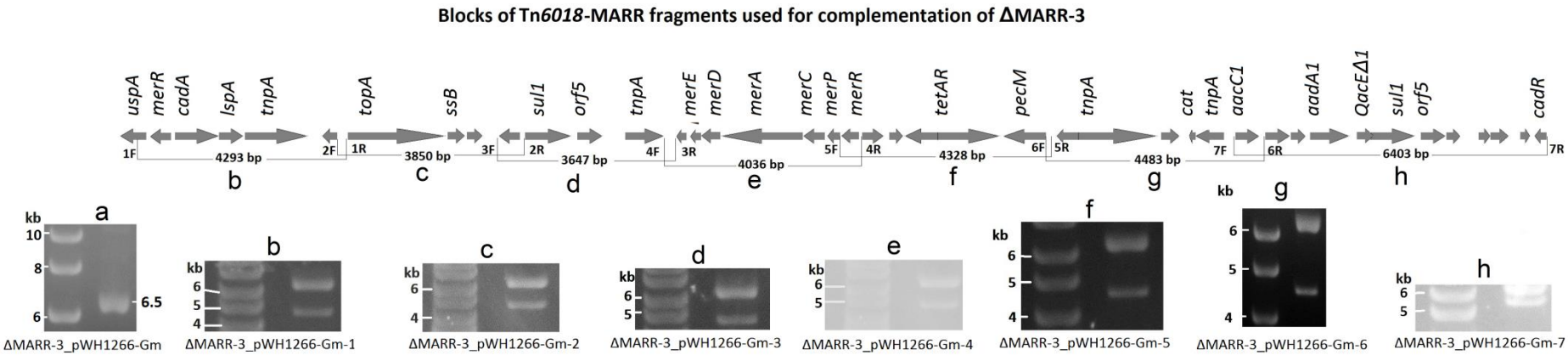

Figure 6.6 Plasmid complementation of ΔMARR-3

Blocks of Tn*6018*-MARR fragments were cloned in ~6.5 kb *E. coli - A. baumannii* shuttle vector pWH1266-Gm (a) to complement spontaneous mutant ΔMARR-3. Plasmids were recovered from complemented ΔMARR-3 strains and digested for confirmation of presence of ~4.3 kb fragment 1 in ΔMARR-3_pWH1266-Gm-1 (b), ~3.9 kb fragment 2 in ΔMARR-3_pWH1266-Gm-2 (c), ~3.7 kb fragment 3 in ΔMARR-3_pWH1266-Gm-3 (d), ~4 kb fragment 4 in ΔMARR-3_pWH1266-Gm-4 (e), ~4.4 kb fragment 5 in ΔMARR-3_pWH1266-Gm-5 (f), ~4.5 kb fragment 6 in ΔMARR-3_pWH1266-Gm-6 (g) and 6.4 kb fragment 7 in ΔMARR-3_pWH1266-Gm-7 (h)

The complemented ΔMARR-3 strains were tested for their antibiotic resistance profile and subsequently used in a head-to-head *in vitro* competition with A424 wild type for fitness comparison.

6.3.7 Effect of Tn*AbaR23* on the phenotype of A424

Antibiotic susceptibility profile of A424 wild type, Tn*AbaR23* deleted mutants and Tn*6018*-MARR complemented strains was assessed for 15 different antibiotics (Table 6.4) using antibiotic discs and e-test strips according to the British Society for Antimicrobial Chemotherapy (BSAC) methods for antimicrobial susceptibility testing guideline version 12,May 2013 (Wootton 2013). For the disc test, the diameter of zone of clearance was measured to the nearest 0.5 mm, values in bold represent the MIC (shown in µg/ml, tested by using Etest strips). The experiment was repeated three times independently. Reference strain NCTC12241 was used as BSAC reference control (Wootton 2013). BSAC version 2013 guideline on zone diameter breakpoints are as follows: for IMP, resistant (R) ≤ 13, intermediate (I) 14 - 24 and susceptible (S) ≥ 25; for AMK, resistant (R) ≤ 18, intermediate (I) 19-20 and susceptible (S) ≥ 21; for gentamicin, resistant (R) ≤ 19 and susceptible (S) ≥ 20; for ciprofloxacin, resistant (R) ≤ 20 and susceptible (S) ≥ 21. BSAC guideline on MIC breakpoint for *Acinetobacter* species: for gentamicin, resistant (R) > 4 and susceptible (S) ≤ 4; for amikacin, resistant (R) > 16, intermediate (I) 16 and susceptible (S) ≤ 8; for imipenem, resistant (R) > 8, intermediate (I) 4-8 and susceptible (S) ≤ 2 and for ciprofloxacin, resistant (R) > 1 and susceptible (S) ≤ 1.

Table 6.4 Antibiotic susceptibility of A424WT and its various mutants

Strain	Zone of inhibition Diameter (mm)														
	TET 10	**IMP 10**	**TOB 10**	**AMP 25**	**CIP 5**	**CTX 30**	**STR 10**	**RIF 2**	**CAR 100**	**AMK 30**	**TMP 2.5**	**CHL 10**	**SUL 25**	**SXT 25**	**GEN 10**
A424WT	6.0	19.0	27.5	6.0	14.5, **4**	6.0	16.5	14.5	6.0	17.0	9.5	6.0	6.0	6.0	25.5
DCO174	15.5	16.5	24.0	6.0	6.0, **> 32**	6.0	10.5	14.0	6.0	12.5	7.5	6.0	27.5	25.0	6.5
DCOA14	20.5	18.5	27.0	6.0	13.5, **4**	6.0	18.5	12.0	6.0	18.5	8.5	6.0	30.5	27.0	8.5
DCOC5-4	19.0	19.5	26.0	6.0	13.5, **3**	6.0	18.0	12.0	6.0	18.0	8.0	6.0	29.5	29.0	9.0
DCOA14-Δ7	20.0	18.5	27.0	6.0	13.0, **4**	6.0	18.0	12.5	6.0	18.0	8.0	6.0	30.0	27.5	24.0
DCOA14-Δ18	20.0	18.0	27.5	6.0	13.5, **4**	6.0	18.0	12.0	6.0	18.0	8.0	6.0	30.0	27.0	25.0
ΔMARR-1	21.5	21.0	28.0	6.0	12.5, **3**	6.0	21.0	10.0	6.0	20.0	9.0	6.0	33.0	26.5	27.0
ΔMARR-3	21.0	20.	27.5	6.0	14.0, **3**	6.0	21.0	16.0	6.0	20.0	9.0	6.0	32.0	27.5	27.0
ΔMARR-3_pWH1266-GM	22.0	18.0	28.5	6.0	15.0	6.0	20.0	15.5	6.0	22.0	9.5	6.0	27.5	30.5	6.0
ΔMARR-3_pWH1266-GM-1	19.0	22.5	29.0	6.0	15.5	6.0	21.0	14.0	6.0	22.0	9.5	6.0	27.0	30.5	6.0
ΔMARR-3_pWH1266-GM-2	20.5	21.5	29.5	6.0	14.5	6.0	20.5	14.5	6.0	22.0	9.0	6.0	28.0	30.0	6.0
ΔMARR-3_pWH1266-GM-3	19.5	19.0	27.5	6.0	15.0	6.0	19.5	16.5	6.0	21.5	9.5	6.0	6.0	30.0	6.0
ΔMARR-3_pWH1266-GM-4	20.0	20.5	27.0	6.0	13.5	6.0	21.0	14.5	6.0	20.5	9.5	6.0	27.5	30.0	6.0
ΔMARR-3_pWH1266-GM-5	7.0	20.0	27.0	6.0	15.5	6.0	20.0	15.0	6.0	22.0	8.0	6.0	29.5	29.5	6.0
ΔMARR-3_pWH1266-GM-6	21.5	21.5	29.0	6.0	15.0	6.0	20.0	15.0	6.0	21.5	9.5	6.0	25.5	30.0	6.0
ΔMARR-3_pWH1266-GM-7	20.0	19.5	28.0	6.0	14.5	6.0	21.0	15.0	6.0	21.5	8.5	6.0	6.0	29.0	6.0
NCTC12241	20.5	35.0	21.5	13.0	30.0	35.0	17.0	6.0	18.0	24.0	26.0	20.0	6.0	29.0	23.0

The full form of abbreviated antibiotics is given in method section. The concentration of antibiotics on the disc is given in µg/ml, values in bold represent antibiotic concentration in µg/ml obtained from E-test. The strain NCTC1224 is used as a reference strain. The Tn*AbaR23* deleted mutant DCO174 was constructed by Kochar *et al.* (2012). The mutants DCOA14 and DCOC5-4 are independent Tn*AbaR23* deleted mutants created in this study. The mutant DCOA14 was rendered markerless to create markerless mutants DCOA14-Δ7 and DCOA14-Δ18. The mutant ΔMARR-1 and ΔMARR-3 are derived from A424 after the spontaneous loss of Tn*6018*-MARR region from Tn*AbaR23* island. The mutant ΔMARR-3_pWH1266-GM is spontaneous mutant ΔMARR-3 carrying the *E. coli - A. baumannii* shuttle vector pWH1266-Gm. The mutants ΔMARR-3_pWH1266-GM-1-7 carry Tn*AbaR23* fragments for complementation.

The antibiotic sensitivity of two new independent Tn*AbaR23* deleted mutants DCOA14 and DCOC5-4 was highly comparable with each other. The expected increased susceptibility towards tetracycline and sulfamethoxazole was observed for Tn*AbaR23* deleted mutant DCO174 reported by Kochar *et al.* (2012) and new independent Tn*AbaR23* deleted mutants DCOA14 and DCOC5-4. This increased susceptibility towards tetracycline and sulfamethoxazole in Tn*AbaR23* deleted mutants could almost certainly be due to the loss of two copies of sulfamethoxazole resistance conferring *sul1* genes and tetracycline resistance conferring determinants *tetA* and *tetR* within Tn*AbaR23* island. The Tn*AbaR23* deleted mutants DCO174, DCOA14 and DCOC5-4 also showed expected increase in gentamicin resistance due to the presence of *aacC1* marker in their chromosomes. Unlike DCO174, none of the two newly generated Tn*AbaR23* deleted mutants DCOA14 and DCOC5-4 show increased resistance towards ciprofloxacin and their diameter of zone of clearance around ciprofloxacin disc was comparable to their parent A424WT. Apart from DCO174, none of the tested mutants exhibited any unusual and unexpected susceptibility towards the above tested 15 different antibiotics.

The markerless Tn*AbaR23* deleted mutants were created by flipping out *aacC1* cassette from Tn*AbaR23* deleted mutant DCOA14. The loss of the *aacC1* cassette in two markerless mutants DCOA14-Δ7 and DCOA14-Δ18 was concurrent with the reversion of gentamicin sensitivity. In effect, the gentamicin susceptibility of markerless mutants DCOA14-Δ7 and DCOA14-Δ18 was comparable to that of their parent DCOA14 and A424 wild type. The tetracycline and sulfamethoxazole sensitivity of markerless Tn*AbaR23* deleted mutants DCOA14-Δ7 and DCOA14-Δ18 was also comparable to their parent DCOA14. The markerless Tn*AbaR23* deleted mutants DCOA14-Δ7 and DCOA14-Δ18 did not show any unusual antibiotic resistance towards the above tested antibiotics.

As discussed earlier in chapter 5, four independent spontaneous mutant viz. ΔMARR-1, ΔMARR-2, ΔMARR-3 and ΔMARR-4 were isolated from A424 wild type. Two representative spontaneous mutant ΔMARR-1 and ΔMARR-3 were tested for their

antimicrobial susceptibility. The spontaneous mutants showed expected increased sensitivity towards sulfamethoxazole and tetracycline almost certainly due to the loss of two copies of sulfamethoxazole resistance conferring *sul1* genes and tetracycline resistance conferring determinants *tetA* and *tetR* from Tn*6018*-MARR region within Tn*AbaR23*. The antibiotic sensitivity of two representative spontaneous mutant ΔMARR-1 and ΔMARR-3 towards rest of the 13 antibiotics tested was highly comparable to their parent A424 wild type.

Antibiotic susceptibility profile of plasmid complemented ΔMARR-3 strain was also highly comparable to their parent ΔMARR-3 except for the expected increased gentamycin resistance due to the presence of gentamicin resistance marker in pWH1266-GM. The Tn*AbaR23* complementation fragment 3 and fragment 7 contains one copy of *sul1* gene. Therefore, sulfamethoxazole sensitive spontaneous mutant ΔMARR-3 when fragments 3 and 7 were complemented in spontaneous mutant strains ΔMARR-3; the sulfamethoxazole resistance was restored as evident from the increased sulfamethoxazole resistance in plasmid complemented strains pWH1266-GM-3 and ΔMARR-3_pWH1266-GM-7. Also, the tetracycline resistance was restored when the spontaneous mutant was complemented with Tn*AbaR23* fragment carrying *tetA/R* genes (fragment 5). The presence of pWH1266-GM plasmids in the ΔMARR-3 strain did not result in unusual and altered antibiotic susceptibility towards the tested antibiotics.

Other than the expected increased susceptibility towards the antibiotics whose determinants were present in the deleted region, the overall antibiotic resistance profile of A424 wild type and various A424 derived mutants created in this study remained almost same. Upon the deletion and complementation of Tn*Aba*R23-associated regions, the overall susceptibility profile of the mutants was found unchanged for the 15 antibiotics tested in this study.

6.3.8 Loss of Tn*AbaR23* does not result in elevated ciprofloxacin resistance

As described earlier in introduction section, the deletion of Tn*AbaR23* was assumed to be associated with the unexpected increase in ciprofloxacin MIC in DCO174 (Kochar *et al.* 2012). The wild type strain A424 and various Tn*AbaR23* deleted mutants created in this study were investigated for their susceptibility towards 15 different antibiotics including ciprofloxacin. It is apparent from Table 6.4 that none of the Tn*AbaR23* deleted mutants created in this study exhibited unexpected resistance or susceptibility towards any of the 15 antibiotics tested. Therefore, no evidence was found to support the possible association of Tn*AbaR23* deletion on the enhanced ciprofloxacin resistance in A424 strain.

6.3.9 No effect of Tn*AbaR23* on growth dynamics

The growth of A424 and its various Tn*AbaR23* deleted mutants in the antibiotic free broth was assessed over a period of 24 h. The growth curve of A424WT and its various Tn*AbaR23* deleted (a) and complemented mutants (b) appeared indistinguishable from each other with no apparent defect in growth dynamics due to mutagenesis (Figure 6.7).

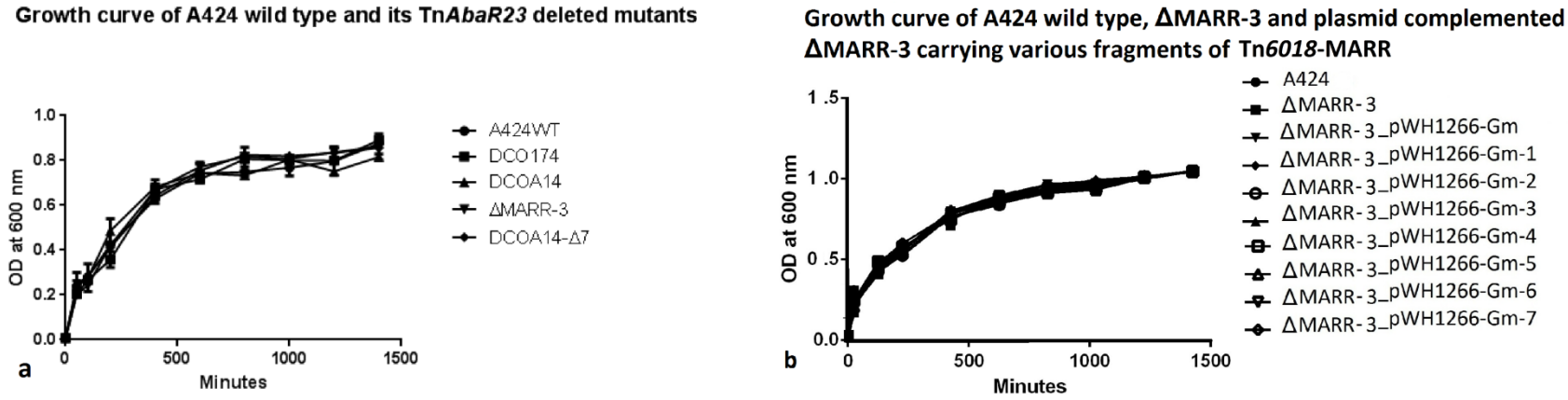

Figure 6.7 Growth curve of the A424WT and its various mutants

The data shown represents the mean value ± standard deviations (error bars) for three independent wells for each strain. No significance difference in the growth dynamics was observed between A424 wild type and its Tn*AbaR23* deleted mutants DCOA14, DCO174 and DCOA14-Δ7 and spontaneous mutant ΔMARR-3 (a) with no significant difference. The growth curve of A424 wild type, and its spontaneous mutant ΔMARR-3 complemented with various fragments of Tn*6018*-MARR appeared indistinguishable and highly comparable (b) no significant difference. Statistical analysis was performed using one-way ANOVA. *P* values less than 0.05 were considered as statistically significant.

6.3.10 No effect of Tn*AbaR23* on biofilm production

The biofilm forming capability of *A. baumannii* has been suggested as a major factor that ensures the persistence and virulence of these bacteria (Gaddy and Actis 2009). Since the biofilm forming capability of A424 has not been know, wild type strain A424 and its various Tn*AbaR23* deleted mutants were tested for their ability to form biofilm *in vitro*.

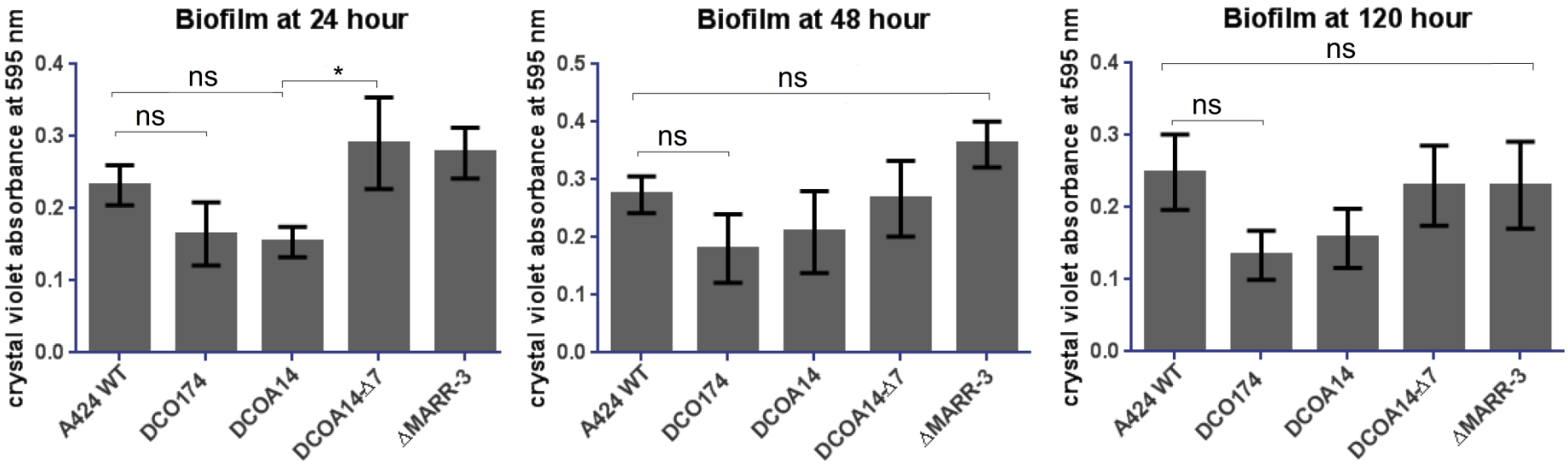

Figure 6.8 Biofilm formed by A424WT and its various Tn*AbaR23* deleted mutants

The amount of biofilm formed was tested at three different time points, 24 h, 48 h and 120 h by measuring the absorbance of dye crystal violet associated with biofilm, LB media without bacteria was used as a negative control. The experiment was repeated three times independently. Error bar represents mean value ± Standard deviation. Statistical analysis was completed by using one-way ANOVA (for the comparison of multiple data sets) and two way ANOVA (for the comparison of two data sets); p values less than 0.05 were considered statistically significant. ns = not significant, * = significant

The markerless mutant DCOA14-Δ7 derived from Tn*AbaR23* deleted *aacC1* marker carrying mutant DCOA14 showed increased biofilm forming ability (p = 0.03) in 24 hour (Figure 6.8). However, no significant difference was observed in the biofilm forming ability of the all the tested strains in 24 h, 48 h and 120 h. Overall, the biofilm forming ability of the strain was not affected upon the deletion of Tn*AbaR23.*

6.3.11 Effect of Tn*AbaR23* on *in vitro* growth fitness

The fitness of A424 wild type and its various Tn*AbaR23* deleted mutants was assessed in an *in vitro* head-to-head competition assay. The population of parent wild type and the mutant was estimated at the beginning of the competition and at specific time points by sampling the culture and plating the appropriate dilution on selective and non-selective plates. The five day head-to-head competition was repeated three times independently. The CFU obtained on both selective and non-selective plates was used to calculate the percentage of wild type and mutant at each time point (T).

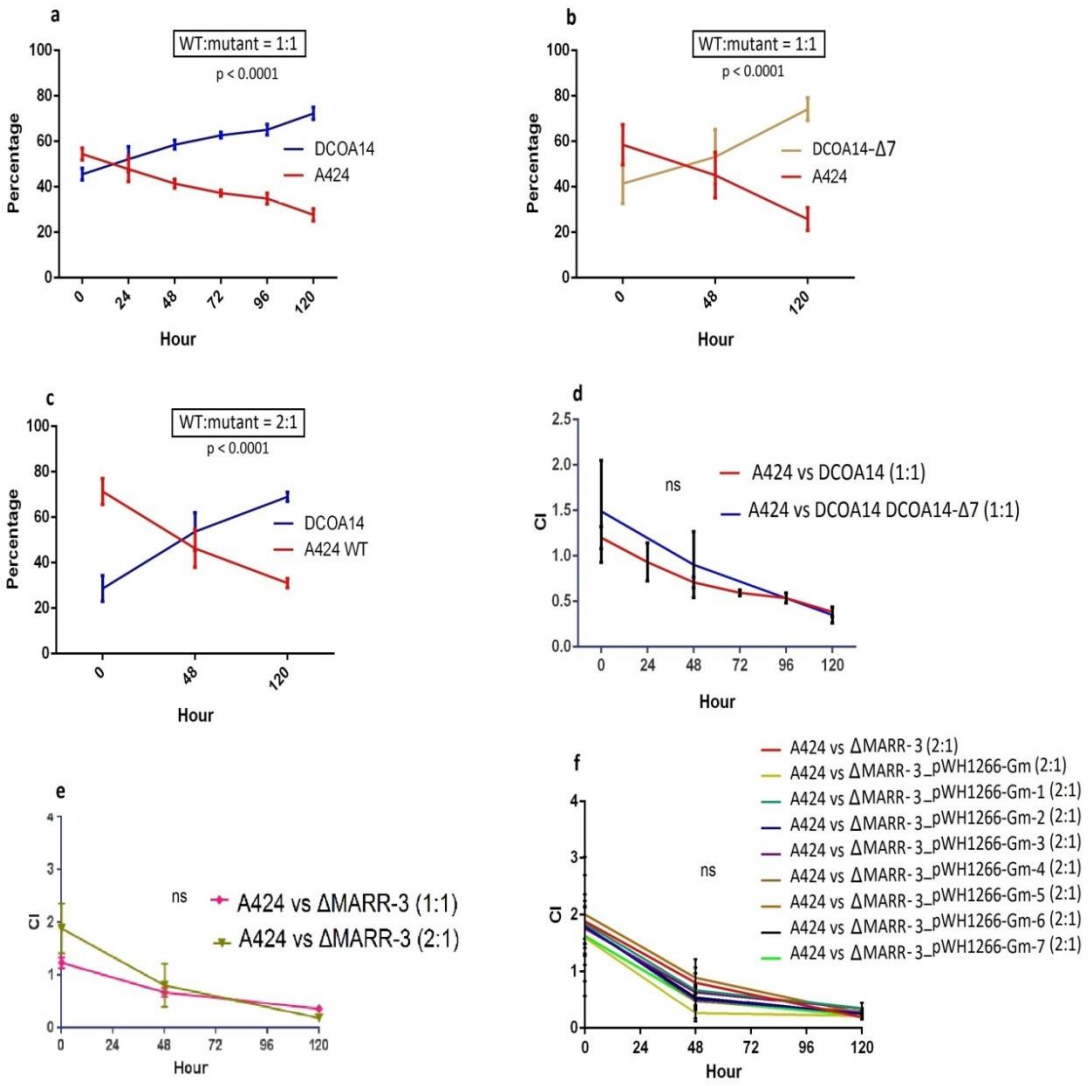

Figure 6.9 Head-to-head *in vitro* competition between A424 wild type and its mutants Head-to-head competition between A424 wild type and its Tn*AbaR23* deleted mutant DCOA14; parent A424 wild type appears less fit when equal number (1:1) of wild type and mutant were competed (a). The parent WT was less fit even in the competition with its Tn*AbaR23* deleted markerless mutant DCOA14-Δ7 (b). The parent A424 appears let fit compared to DCOA14 even when the starting population of wild type was double than mutant (c). Competitive index (CI) is shown for the competition between WT and mutants DCOA14 and DCOA14-Δ7 (d), CI 1 meaning equal number of WT and mutant, CI 2 means the number of WT is twice the number of mutant. The Tn*6018*-MARR deleted spontaneous mutant also appeared fitter compared to its wild type parent A424 even when the starting population of mutant was half of the A424 wild type (e). The MARR complemented strains maintained their *in-vitro* fitness in a head-to-head competition with their parent A424 wild type (f). Percentage of bacteria at each time point is plotted in Y axis and time in hour plotted in X – axis.

The Figure 6.9 shows the *in vitro* competitive fitness of A424 wild type and its various mutants. The parent A424 was out-competed by Tn*AbaR23* deleted mutant DCOA14 (a), Tn*AbaR23* deleted markerless DCOA14-Δ7 (b) and Tn*6018*-MARR deleted spontaneous mutant ΔMARR-3 (e) when equal number of wild type and mutants were present at the beginning of competition. The mutants DCOA14 and ΔMARR-3 exhibited enhanced competitive fitness in a head-to-head competition with A424 wild type even when their starting number was half the number of wild type (c and e). The presence of *aacC1* marker did not seem to affect the overall competitive fitness since no significant difference in CI was observed for DCOA14 and DCOA14-Δ7 in their head-to-head competition with A424 (d). Importantly, the presence of Tn*6018*-MARR fragments in plasmid complemented ΔMARR-3 mutants did not alter the fitness of complemented strains and no significant difference in CI was observed between empty ΔMARR-3 strains and plasmid complemented strains (f). These data suggests that the possession of intact Tn*AbaR23* results in decreased *in vitro* growth fitness, however, it was not possible to pin point the region within Tn*AbaR23* that would impose the observed physiological cost in A424.

6.3.12 Effect of Tn*AbaR23* on virulence

The virulence of A424 wild type and its various Tn*AbaR23* deleted mutants was compared in a killing assay using the sixth instar larva of *G. mellonella*. Each larva was injected with a 20 µl of bacterial suspension and the mortality of the larva was monitored over a period of 5 days. At the end of the experiment, the surviving larvae were disposed. Before commencing the killing assay, the appropriate dose of bacterial inoculum was determined for A424 wild type strain.

6.3.12.1 Determination of inoculum size

The appropriate bacterial load of A424 wild type for use in a five-day *G. mellonella* killing assay was determined by challenging *Galleria* larva with various loads of the A424 wild type strain. Bacterial density of 10^4 colony forming unit (CFU) in 20 µl volume of inoculum was found to kill the larva consistently over a period of five days (Figure 6.10). A higher bacterial load of 10^5 bacteria per larva was found to kill around 70% of larva in 24 hours. However, a lower bacterial load of 10^3 bacteria per larva did not kill any larva at all. Therefore, in the *G. mellonella* killing assay, bacterial load of 10^4 in 20 µl inoculum was used to test the virulence of A424 wild type and its various mutants.

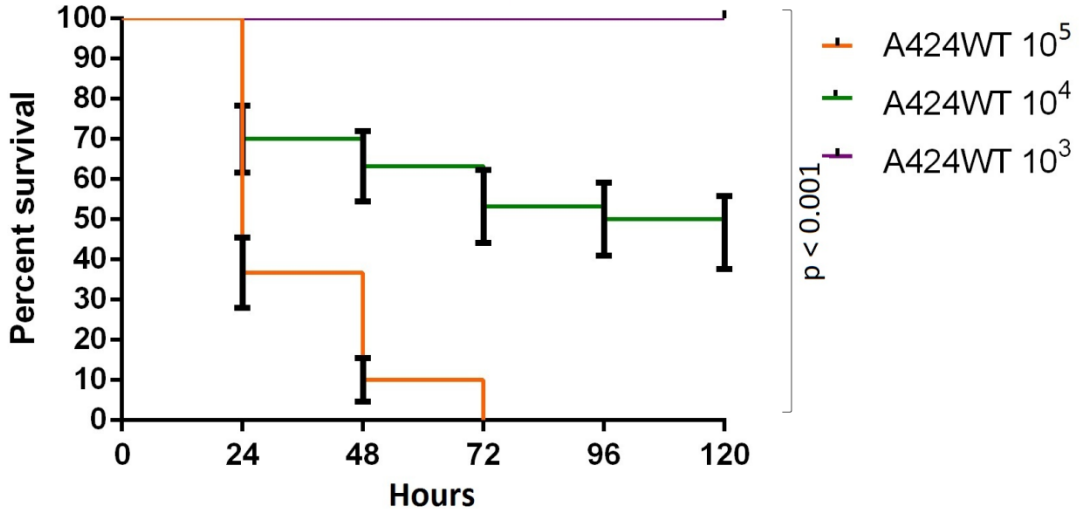

Figure 6.10 Determination of inoculum size for *Galleria* killing assay

A group of 15 larva was injected with 10^3, 10^4 and 10^5 CFU of A424 wild type. Death of the larva was recorded over a period of 5 days. Three independent experiments consisting of 15 larvae per treatment group were performed using different batches of larvae. The inoculum containing 10^5 bacteria appeared to kill more than 70% of larva in 24 h. The inoculum containing 10^3 bacteria on the other hand did not kill any larva at all over a period of 120 h or 5 days. Consistent killing was observed for inoculum containing 10^4 bacteria. All the surviving larvae were censored at the end of the experiment.

6.3.12.2 Comparison of virulence

In the *Galleria* killing assay, a group of 15 larva per strain were injected with 5.0 – 5.5 × 10^4 CFU of bacteria. Death of the larva was recorded over a period of 5 days. Three independent experiments consisting of 15 larvae per treatment group were performed using different batches of larvae.

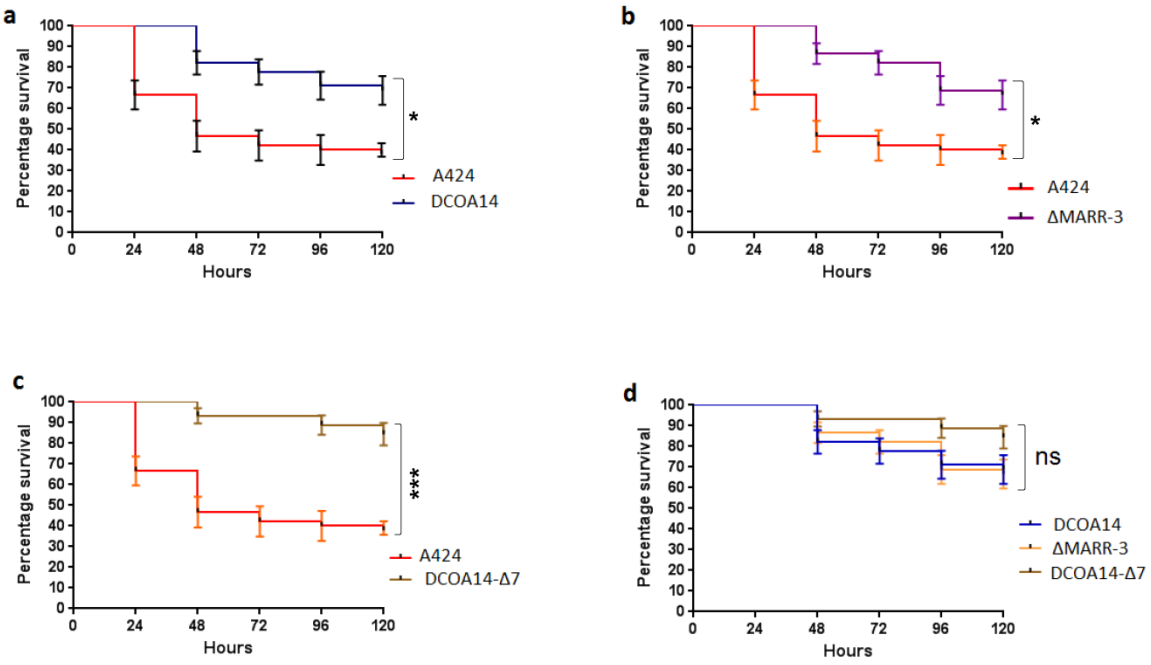

Figure 6.11 Effect of Tn*AbaR23* on virulence

The wild type parent A424 appeared virulent compared to its Tn*AbaR23* deleted mutants DCOA14, Tn*AbaR23* deleted markerless mutant DCOA14-Δ7 and Tn*6018*-MARR deleted spontaneous mutant ΔMARR-3 (a, b and c). Figure d is a pooled virulence data for A424 wild type, DCOA14, Tn*AbaR23* deleted markerless mutant DCOA14-Δ7 and Tn*6018*-MARR deleted spontaneous mutant ΔMARR-3. Horizontal lines represent the percentage of *G. mellonella* larvae surviving after inoculation with each bacterial strain at the indicated time point. The asterisk (***) represents a highly significant p value which is less than 0.001, ns = not significant.

The DCOA14 and its markerless version DCOA14-Δ7 appeared significantly less virulent compared to their parent A424 wild type (Figure 6.11 a and c). The deletion of Tn*6018*-MARR also appears to attenuate the virulence of the strain and a decrease in virulence

was observed in ΔMARR-3 strains (Figure 6.11b). The virulence of spontaneous mutant ΔMARR-3, Tn*AbaR23* island deleted mutants DCOA14 and its markerless derivative DCOA14-Δ7 was highly comparable (Figure 6.11d) and the difference in their virulence was not significant statistically. Since the attenuation of virulence was observed in strains carrying completely or partially deleted Tn*AbaR23*, the present *Galleria* killing assay strongly supports the idea that Tn*AbaR23* contributes positively towards the virulence of A424.

6.3.13 DCO174 harbour mutation in AdeABC regulator gene *adeS*

The presence of a single nucleotide polymorphism [SNP] in the AdeABC regulator gene *adeS* was reported by Crosatti *et al.* (unpublished data, 2013). The nucleotide and amino acid sequence comparison of *adeS* in DCO174 [1] and A424 showed that the G to A substitution in DCO174 *adeS* was a non-synonymous SNP located at the 178th position of *adeS* gene sequence and it resulted in a substitution of an aspartic acid to asparagine at position 60 of the 356 amino acid AdeS protein[2] (Figure 6.12). Importantly, the new independent Tn*AbaR23* deleted mutants DCOA14 and DCOC5-4 did not possess any SNP in their *adeS* genes. In the following sections the mutation in the *adeS* gene in DCO174 is referred as *adeS*^{G178A}.

[1] The sequence of DCO174 was obtained from Crosatti *et al.* unpublished data 2013

[2] The findings from this work are reported in the third year project 2015 submitted by Joseph Jack Wanford to the School of Biological Sciences, University of Leicester

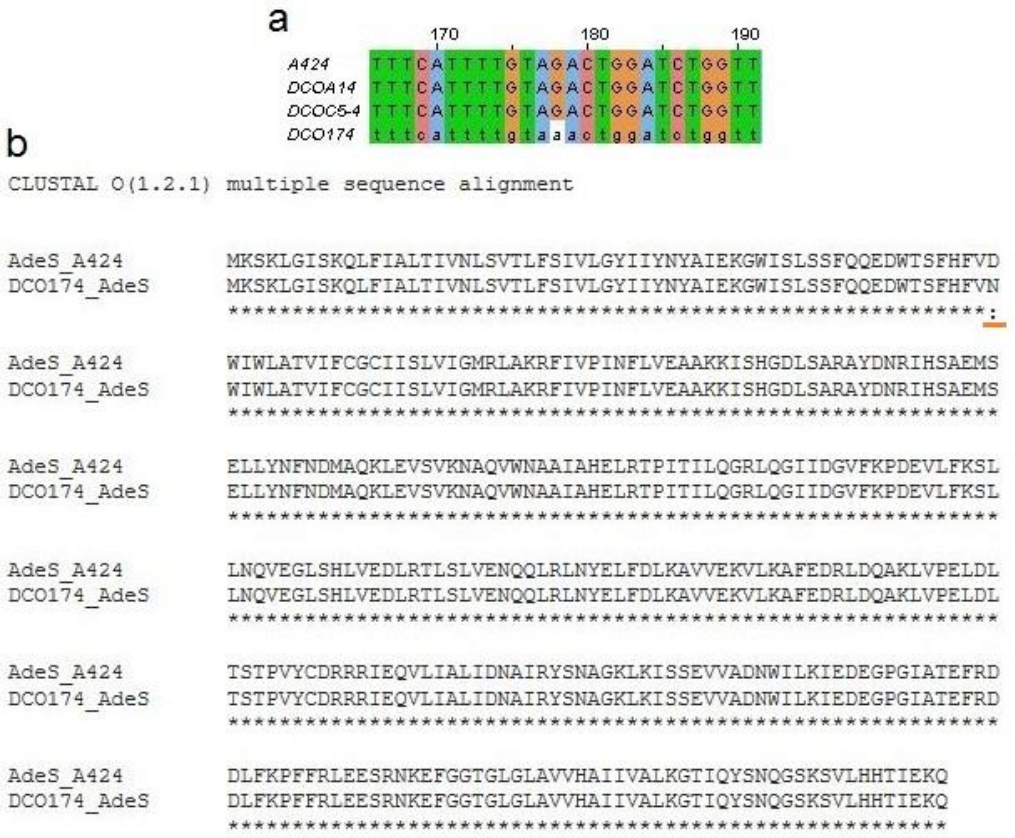

Figure 6.12 A non-synonymous SNP in DCO174
Pairwise multiple alignment of nucleotide sequence of A424 wild type, Kochar *et al.* (2012) reported DCO174 and two independent Tn*AbaR23* deleted mutants DCOA14, DCOC5-4 created in this study showing the position of G→A single nucleotide polymorphism (SNP) in *adeS* gene (a). New independent Tn*AbaR23* deleted mutants DCOA14, DCOC5-4 and parent strain A424 did not harbour any SNP at 178th position in *adeS* gene. The amino acid sequence of AdeS from A424 wild type and DCO174 shows the SNP to be non-synonymous. The SNP in *adeS* gene of DCO174 resulted in the substitution of aspartic acid to asparagine at position 60 of AdeS underlined (b).

6.3.14 Replacement of *adeS*^{WT} with *adeS*^{G178A} caused enhanced ciprofloxacin resistance

The wild type *adeS* allele is referred to as *adeS*^{WT} and the mutant allele in DCO174 as adeSG178A from this position onwards. To check whether *adeS*^{G178A} alone was sufficient to lead to higher level of ciprofloxacin resistance as observed in DCO174, the *adeS*^{WT} in A424 wild type and its various Tn*AbaR23* deleted mutants was replaced with

adeS^{G178A} in an allelic exchange using a suicide deletion vector pJW-1[3]. An allele-specific PCR assay was established to distinguish between *adeS*^{G178A} and *adeS*^{WT}. Successful gene replacement was achieved in A424 wild type, DCOA14-Δ7 and ΔMARR-3 background.

In order to examine the effect *adeS*^{G178A} on the overall resistance profile of the mutants, the susceptibility of *adeS*^{G178A} carrying mutant strains towards different antibiotics was tested (Table 6.5). The ciprofloxacin MIC values for the strain carrying *adeS*^{WT} were 3 - 4 μg/ml, while those of the matching isogenic strains carrying the *adeS*^{G178A} were 24 - 32 μg/ml. Compared to their parent strains, all the *adeS*^{G178A} carrying mutant strains showed 6-8 fold increase in MIC. The MIC of the wild type reverted mutants was comparable to their parent strains indicating a reversion to ciprofloxacin susceptibility in the absence of *adeS*^{G178A}.

The resistance profile of the *adeS*^{G178A} carrying mutant strains, reverted wild type strains and their matching parent strains was highly comparable. Moreover, unexpected antibiotic susceptibility or resistance towards any of the tested antibiotics was not observed in any of the tested strains.

The genotypes of the strains in terms if *adeS* allele are given in strain table in method section. In brief, the genotypes of various mutant strains are as follows:
A424: wild type parent *adeS*^{WT}
DCO174: Tn*AbaR23* deleted mutant of A424 (Kochar *et al.* 2012), *adeS*^{G178A}
DCOA14: Tn*AbaR23* deleted mutant derived from A424, *adeS*^{WT}
DCOA14-Δ7: Tn*AbaR23* deleted markerless mutant derived from DCOA14, *adeS*^{WT}
ΔMARR-3: Tn*6018*-MARR deleted spontaneous mutant of A424, *adeS*^{WT}
A424_SNP_DCO: A424 mutant, *adeS*^{G178A}
Δ7_SNP_DCO: DCOA14-Δ7 mutant, *adeS*^{G178A}
Δ3_SNP_DCO: ΔMARR-3 mutant, *adeS*^{G178A}
A424 rev: A424 mutant reverted to *adeS*^{WT} post sucrose counter selection

[3] The suicide deletion vector pJW-1 was jointly constructed by Sapkota NP and Wanford JJ. The *adeS*^{G178A} from DCO174 was cloned at the *NotI* and *XbaI* site of suicide vector pJTOOL-3-Apra.

Δ7_rev: DCOA14-Δ7 mutant reverted to *adeS*^{WT} post sucrose counter selection

Δ3_rev: ΔMARR-3 mutant reverted to *adeS*^{WT} post sucrose counter selection

For the disc test, the diameter of zone of clearance was measured to the nearest 0.5 mm, values in bold represent the MIC (shown in μg/ml, tested by using Etest strips). The experiment was repeated three times independently. Reference strain NCTC12241 was used as BSAC reference control (Wootton 2013). The full form of antibiotics abbreviated below is given in method section. BSAC version 2013 guideline on zone diameter breakpoints are as follows: for IMP, resistant (R) ≤ 13, intermediate (I) 14 - 24 and susceptible (S) ≥ 25; for AMK, resistant (R) ≤ 18, intermediate (I) 19-20 and susceptible (S) ≥ 21; for gentamicin, resistant (R) ≤ 19 and susceptible (S) ≥ 20; for ciprofloxacin, resistant (R) ≤ 20 and susceptible (S) ≥ 21. BSAC guideline on MIC breakpoint for *Acinetobacter* species: for gentamicin, resistant (R) > 4 and susceptible (S) ≤ 4; for amikacin, resistant (R) > 16, intermediate (I) 16 and susceptible (S) ≤ 8; for imipenem, resistant (R) > 8, intermediate (I) 4-8 and susceptible (S) ≤ 2 and for ciprofloxacin, resistant (R) > 1 and susceptible (S) ≤ 1.

Table 6.5 Antibiotic susceptibility profile of strains carrying *adeS*^{WT} and *adeS*^{G178A}

Strain	Zone of inhibition Diameter (mm)										
	TET	**IMP**	**TOB**	**CIP**	**STR**	**RIF**	**AMK**	**TMP**	**SUL**	**SXT**	**GEN**
	10	**10**	**10**	**5**	**10**	**2**	**30**	**2.5**	**25**	**25**	**10**
A424WT	6.0	19.0	27.5	14.5, **4**	16.5	14.5	17.0	9.5	6.0	6.0	25.5
DCO174	15.5	16.5	24.0	6.0, **> 32**	10.5	14.0	12.5	7.5	27.5	25.0	6.5
DCOA14	20.5	18.5	27.0	13.5, **4**	18.5	12.0	18.5	8.5	30.5	27.0	8.5
DCOA14-Δ7	20.0	18.5	27.0	13.0, **4**	18.0	12.5	18.0	8.0	30.0	27.5	24.0
ΔMARR-3	21.0	20.	27.5	14.0, **3**	21.0	16.0	20.0	9.0	32.0	27.5	27.0
A424_SNP_DCO	6.0	19.5	26.5	**24**	17.0	16.0	16.5	10.5	6.0	6.0	28.5
Δ7_SNP_DCO	23.0	20.5	28.0	**24**	19.0	14.5	17.0	9.0	33.0	29.5	24.0
Δ3_SNP_DCO	22.5	20.5	29.5	**> 32**	23.0	17.5	21.0	9.5	32.0	27.0	27.0
A424_rev	6.5	18.0	26.5	**2**	16.0	14.0	17.5	9.0	6.0	6.0	25.0
Δ7_rev	21.0	17.0	25.5	**3**	18.5	12.0	19.0	8.5	32.0	27.0	25.5
Δ3_rev	21.0	22.0	27.0	**3**	23.5	15.0	21.5	9.0	33.0	27.0	28.5
NCTC12241	20.5	35.0	21.5	30.0	17.0	6.0	24.0	26.0	6.0	29.0	23.0

The full form of abbreviated antibiotics is given in method section. The concentration of antibiotics on the disc is given in µg/ml, values in bold represent antibiotic concentration in µg/ml obtained from E-test. The strain NCTC1224 is used as a reference strain. The parent A424 wild type carrying mutant *adeS*^{G178A} *adeS* allele (A424_SNP_DCO) shows 6 folds increase in ciprofloxacin resistance. A 6 – 8 folds increases in ciprofloxacin resistance observed in strains Δ7 and Δ3 where the wild type *adeS*^{WT} allele has been replaced by mutant *adeS*^{G178A} allele.

6.3.15 Fitness and virulence phenotypes of DCO174

The competitive growth fitness and virulence phenotype of DCO174 was studied in an *in vitro* head-to-head competition assay and *Galleria* killing assay as described above. The deletion of Tn*AbaR23 adeS*^{G178A} in DCO174 did not altered its biofilm forming ability (Figure 6.8) and growth dynamics (Figure 6.7). However, the competitive fitness of DCO174 was found markedly reduced in an *in vitro* head-to-head competition with its parent A424. The mutant DCO174 was outcompeted by A424 wild type even when population of DCO174 was nine times more than that of A424 at the beginning of competition (Figure 6.13b). The Two-way ANOVA showed no significant difference in the CI when the starting population of DCO174 was equal to or 9 times greater than A424 wild type (Figure 6.13d).

The Tn*AbaR23* deleted isogenic mutants DCOA14 and DCOA14-Δ7, on the other hand, exhibited significantly enhanced fitness in a head-to-head competition with their parent A424 wild type (Figure 6.13c). Compared to their parent A424 wild type, the Tn*AbaR23* deleted mutants DCOA14, DCOA14-Δ7 and Tn*AbaR23* truncated mutant ΔMARR-3 exhibited significantly ($p < 0.001$) attenuated virulence in *Galleria* killing assay (Figure 6.11). Surprisingly, DCO174 did not exhibit attenuated virulence in *Galleria* killing assay. Moreover, the virulence of A424 wild type and DCO174 appeared highly comparable in the *Galleria* killing assay (Figure 6.14).

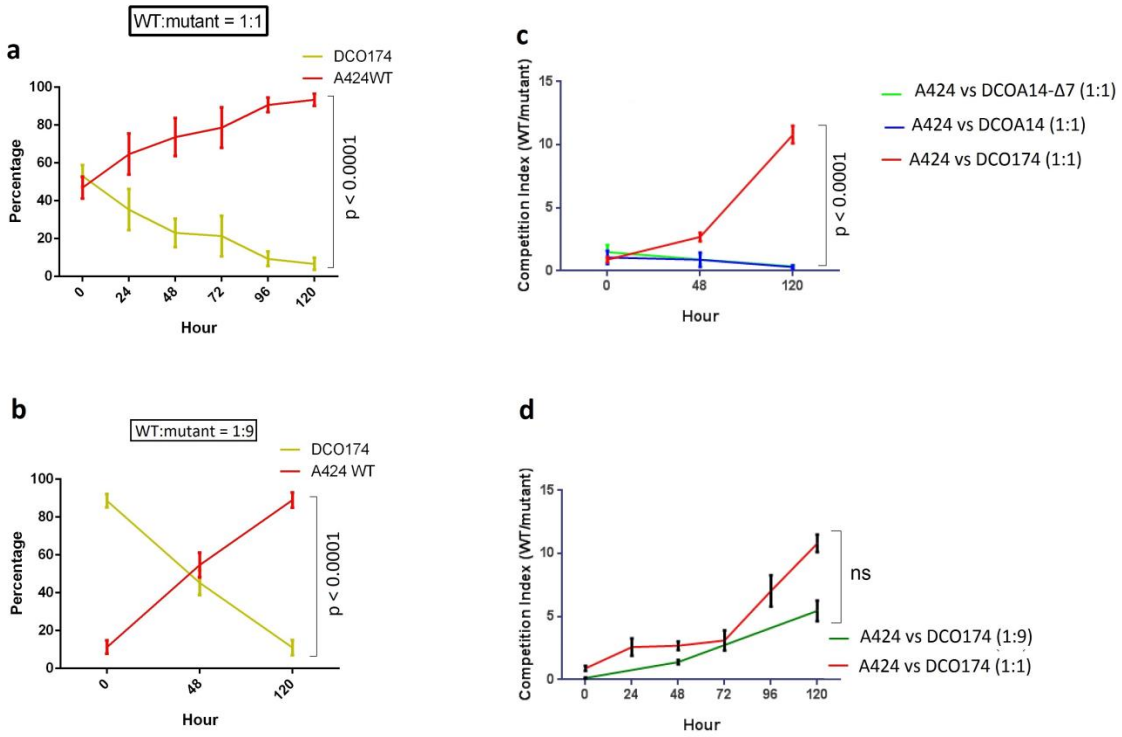

Figure 6.13 *In vitro* competitive fitness of DCO174

Head-to-head competition between A424 wild type and its Tn*AbaR23* deleted mutants DCO174, DCOA14 and markerless mutant DCOA14-Δ7. The parent A424 wild type appears fitter compared to DCO174 when equal number (1:1) of wild type and mutant were competed (a). The parent WT out-grows the DCO174 mutant even when the starting population of mutant DCO174 was 9 times more than A424 wild type (b). Competitive index (CI) is shown for the competition between WT and mutants DCOA14, DCOA14-Δ7 and DCO174 when equal number of wild type and mutants were present at the beginning of the competition (c). The value of CI less than 1 means less A424 wild type and the value of CI more than 1 means more A424 wild type. Percentage of bacteria at each time point is plotted in Y axis and time in hour plotted in X – axis; ns = not significant

Compared to the Tn*AbaR23* deleted mutants DCOA14 and its markerless version DCOA14-Δ7, the Kochar et al (2012) reported DCO174 appeared significantly less fit as shown in the above Figure 6.13.

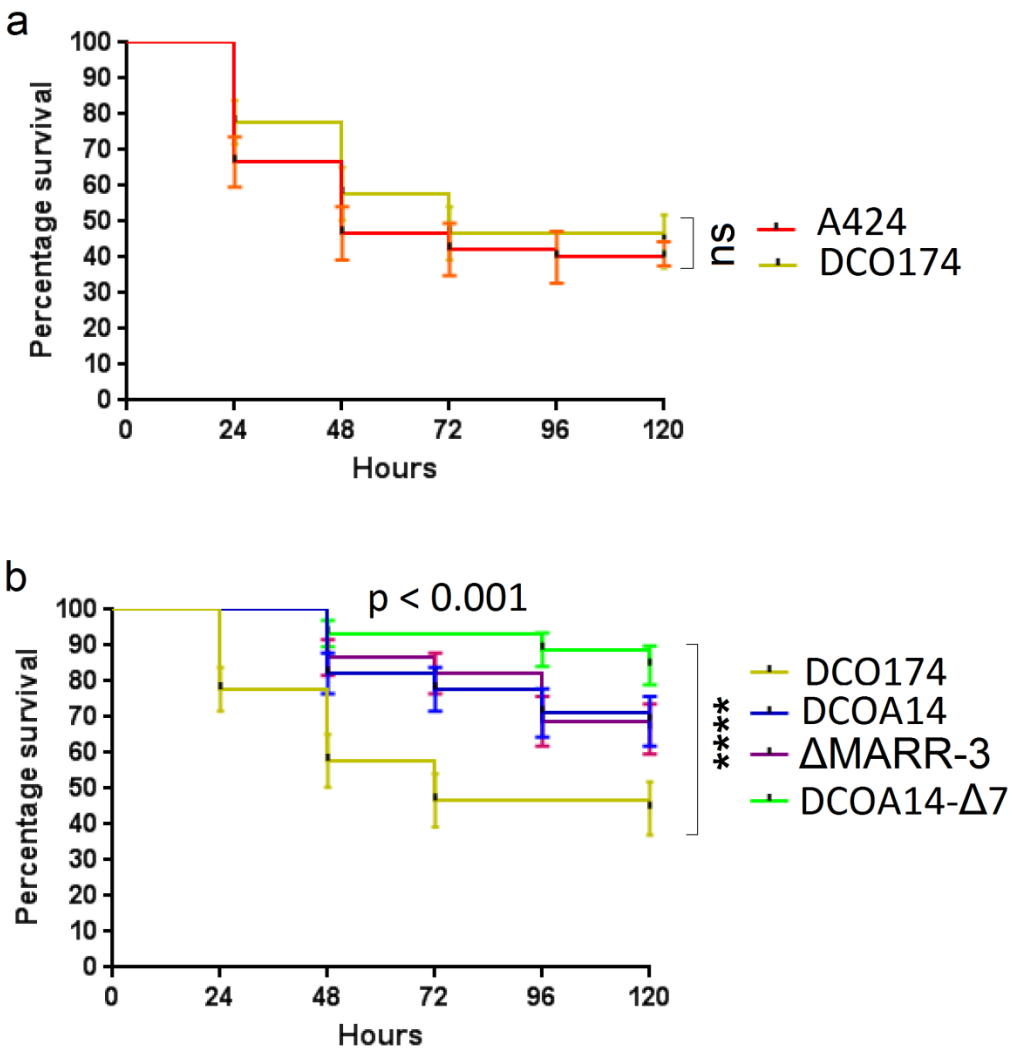

Figure 6.14 Virulence of DCO174

A group of 15 larva per sample was injected with 5.0 – 5.5 $\times 10^4$ CFU of bacteria. Death of the larva was recorded over a period of 5 days. Three independent experiments consisting of 15 larvae per treatment group were performed using different batches of larvae. Horizontal lines represent the percentage of *G. mellonella* larvae surviving after inoculation with each bacterial strain at the indicated time point. The virulence of DCO174 appeared comparable to the virulence of A424 wild type (a). Compared to other Tn*AbaR23* deleted mutants like DCOA14, DCOA14-Δ7 and ΔMARR-3, the mutant DCO174 showed enhanced virulence (b). The asterisk (****) $p < 0.0001$. Statistical analysis was performed using Mantel-cox test. ns = not significant

6.3.16 Growth dynamics in *adeS*^{G178A} mutants

The growth curve of the mutant strains carrying *adeS*^{G178A} was compared to their counterparts carrying wild type *adeS*^{G178A} allele (Figure 6.15). The non-synonymous SNP at *adeS* did not exhibit any growth defect in mutants as apparent by the growth curve below.

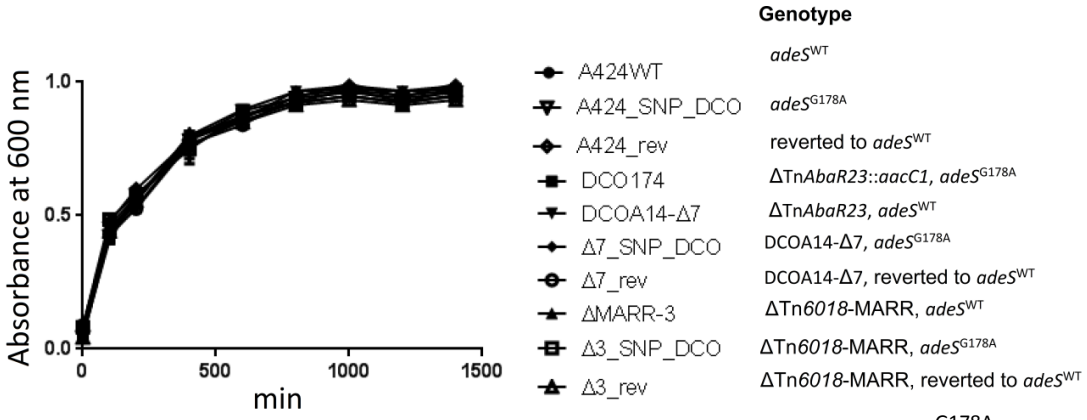

Figure 6.15 Growth curve of A424 wild type and its mutants carrying *adeS*^{G178A}

The replacement of wild type *adeS*^{WT} with mutant *adeS*^{G178A} did not pose any growth defect as shown by the growth curve. The *adeS* genotype of the strains is shown next to their names. The data shown represents the mean value ± standard deviations for three independent wells for each strain. No significance difference in the growth dynamics was observed upon the swapping of *adeS*^{G178A} with *adeS*^{WT} in A424 wild type and various mutants Tn*AbaR23* deleted mutants.

6.3.17 Biofilm formation in *adeS*^{G178A} mutant

The biofilm forming ability of various A424 derived mutants carrying normal and mutant *adeS* allele was compared at 24 h, 48 h and 120 h. The biofilm forming ability of the strains carrying mutant allele *adeS*^{G178A} was comparable to that of the strains carrying wild type allele *adeS*^{WT} (Figure 6.16).

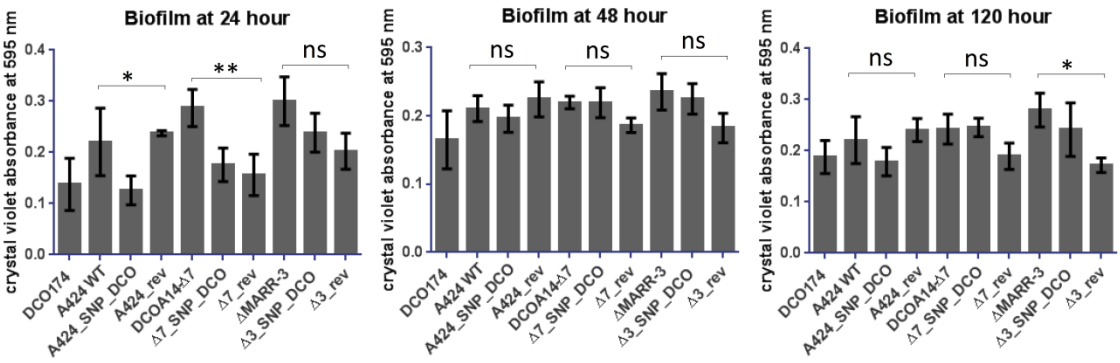

Figure 6.16 Biofilm formation by *adeS*^{G178A} and their parent strains
At 24 h, the amount of biofilm formed by A424 WT and WT reverent A424 was significantly higher compared to A424 carrying *adeS*^{G178A}. Also, for the Tn*AbaR23* deleted markerless mutant DCOA14-Δ7, strain carrying the wild type allele was found to form significantly higher biofilm compared to the one carrying mutant allele at 24 h. No significant difference in biofilm forming ability was seen in the tested strains at 48 h and at 120 h. The amount of biofilm formed was tested at three different time points, 24 h, 48 h and 120 h by measuring the absorbance of dye crystal violet associated with biofilm. The experiment was repeated three times independently. The symbol asterisk (*) represents p = 0.03, ** represents p = 0.009, ns = not significant

6.3.18 In vitro competitive fitness in *adeS*^{G178A} mutants

Head-to-head *in vitro* competition between A424 wild type and its various mutants carrying *adeS*^{G178A} was performed by mixing the wild type and mutant in 1:1. The mutant DCO174 showed reduced *in vitro* fitness compared to all tested strains. The swapping of *adeS*^{G178A} in the Tn*AbaR23* deleted markerless mutant DCOA14-Δ7 and Tn*6018*-MARR deleted ΔMARR-3 strain did not alter their competitive fitness.

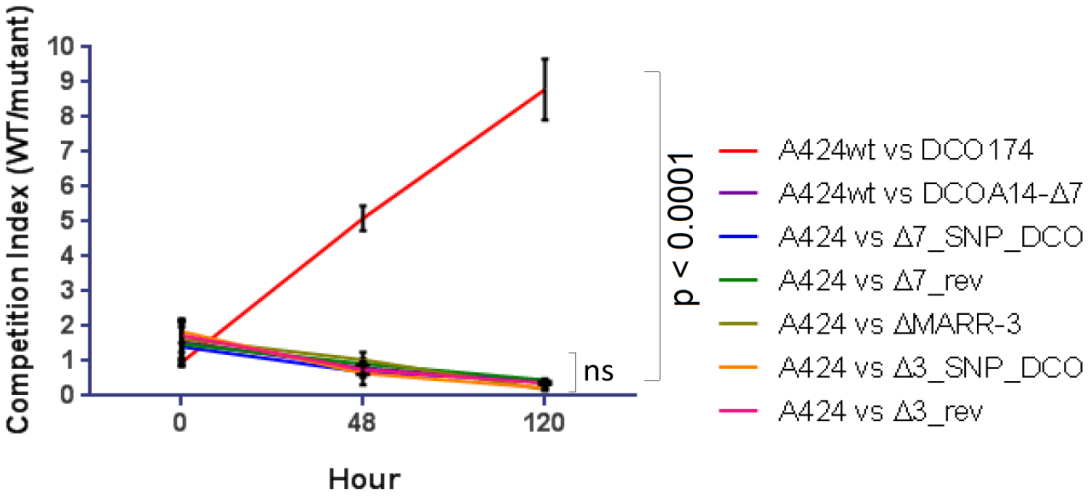

Figure 6.17 *In vitro* competitive fitness of A424 wild type with various mutants carrying *adeS*^{G178A}

Equal number of A424 wild type and mutants were mixed at the beginning of the experiment. The percentage of bacteria at each time point is plotted in Y axis and time in hour plotted in X – axis. The presence of mutant *adeS*^{G178A} allele was not sufficient for the reversion of *in vitro* competitive fitness of the Tn*AbaR23* deleted strains derived from A424. Statistical analysis was completed using One-way ANOVA.

6.4 Discussion and conclusion

6.4.1 New Tn*AbaR23* deleted mutants with stable genetic configuration created

In this study, independent Tn*AbaR23* deleted mutants DCOA14 and DCOC5-4 were created from new independent SCOs using 6.25 µg/ml gentamicin during the selection procedure. The genetic configuration of DCOA14 and DCOC5-4 remained unchanged despite repeated subculture as evident by PFGE profile. The mutant DCOA14 was used as a representative Tn*AbaR23* deleted mutant in the subsequent phenotypic studies.

6.4.2 Markerless Tn*AbaR23* deleted mutants created using pFLP2-derived plasmid

The FRT flanked *aacC1* cassette in DCOA14 was excised in flipase mediated excision employing pFLP2_*sul1*. However, the plasmid pFLP2_*sul1* was only transiently stable in markerless DCOA14-Δ7 and it was completely lost from the cell during repeated

growth. This observation was consistent with previous report regarding the instability of pFLP2 plasmid in *A. baumannii* ATCC17978 (Isberg *et al.* 2012). The use of pFLP2-*sul1* was reproducible and highly successful in the excision of FRT-flanked cassette in DCOA14. Therefore, despite its apparent instability in *A. baumannii*, the protocol developed in this study for the flipase-mediated excision of FRT flanked cassette in *A. baumannii* is highly recommended because it is a one-step, easy to use technique that does not require the downstream curing of plasmid.

6.4.3 Effect of Tn*AbaR23* on the phenotypes of the strain A424

The only significant impact on resistance phenotype following loss of Tn*AbaR23* or Tn*6018*-MARR was observed as a reversion to sulphamethoxazole and tetracycline susceptibility. Kochar (2012) suggested that the loss of Tn*AbaR23* could result in elevated ciprofloxacin resistance. This does not appear to be the case as unusual antibiotic resistance or susceptibility was not observed in any of the Tn*AbaR23* deleted mutants created in this study.

The phenotype of various Tn*AbaR23* deleted mutants created/isolated during this work was assessed using various *in vitro* and *in vivo* assays. The head-to-head *in vitro* growth competition assay clearly and reproducibly demonstrated that the DCOA14, DCOA14-Δ7 and ΔMARR-3 mutants were significantly 'fitter' ($p < 0.001$) than their parent A424 wild type. Since enhanced *in vitro* competitive fitness was observed in A424 mutants upon the loss of Tn*AbaR23* and Tn*6018*-MARR, it is possible that the Tn*6018*-MARR region within Tn*AbaR23* is exerting the cost that leads to decreased *in vitro* competitive fitness observed in A424 wild type. This finding is in agreement with those reported by (Lopez-Rojas *et al.* 2011, Guo *et al.* 2012) where acquisition of the resistance determinants was found associated with fitness cost. Interestingly the, complementation of ΔMARR-3 with blocks of MARR region was not sufficient to diminish the *in* vitro fitness of ΔMARR-3. The reason for this is not clear but it is possible that the physiological cost that ultimately leads to a reduced fitness is exerted by an intact Tn*6018*-MARR region rather than its individual genes and determinants. A

further research involving the capture of circular Tn*6018*-MARR for complementation of ΔMARR-3 mutants could help pin point the determinants posing energetic or physiological costs and understand the molecular mechanism that leads to reduced competitive growth fitness in A424 strain.

Attenuated virulence was observed in Tn*AbaR23* and Tn*6018*-MARR deleted mutant in the *G. mellonella* killing assay as determined by the survival plots and percentage survival over a period of five days. In reviewing the literature, no data was found that on the association of AbaR islands with virulence in *A. baumannii*. However, prior studies have noted the attenuation of virulence upon the deletion of pathogenicity islands in *Pseudomonas aeruginosa* strains (Miyata *et al.* 2003, Harrison *et al.* 2010) Although the possession of the Tn*AbaR23* appears to be energetically and/or physiologically costly for A424 as evident in competitive fitness comparison, the preservation of this island in its intact form seems to be important for the strain in order to maintain its virulence-associated phenotypes. Further research should be taken to understand the underlying mechanism by which Tn*6018*-MARR and Tn*6018*-MARR borne determinants enhances the virulence of A424 strain.

The review on *A. baumannii* biofilm (Gaddy and Actis 2009) suggests that the ability to form biofilm could enhance the virulence in pathogenic *A. baumannii* strains. Despite showing reduced virulence upon the deletion of Tn*AbaR23*, no significant difference was observed regarding the biofilm forming capability in A424 wild type and isogenic Tn*AbaR23* deleted mutants. Based on the findings of this work, while preliminary, it is possible to hypothesize that AbaR islands specifically contribute positively to antibiotic resistance, virulence and/or other environmental survival traits despite the associated physiological costs.

6.4.4 DCO174 an unusual Tn*AbaR23* deleted mutant of A424

As speculated by Kochar *et al.* (2012), the genome analysis of DCO174 showed a non-synonymous SNP in the regulatory gene of efflux pump AdeABC where a substitution of nucleotide G to A at 178th position of *adeS* resulted in substitution of aspartic acid to asparagine in a region of AdeS protein devoid of identified functional domains. In this study, the *adeS*^{WT} allele was replaced with mutant *adeS*^{G178A} allele in A424 wild type and various Tn*AbaR23* deleted strains using an allelic exchange protocol. The mutant strains carrying *adeS*^{G178A} showed 6-8 folds increase in ciprofloxacin MIC. The reversion of ciprofloxacin MIC in the DCOs which reverted to wild type post sucrose-counter selection suggests that the observed heightened ciprofloxacin MIC in *adeS*^{G178A} mutants created in this study was not due to the genetic stress, antibiotic exposure or any alternative mechanisms. It can thus be suggested that the presence of *adeS*^{G178A} on its own was sufficient to lead to elevated level of ciprofloxacin resistance.

The Tn*AbaR23* deleted mutant DCO174 exhibited markedly reduced fitness in a head-to-head competition with its parent A424 wild type. Various Tn*AbaR23* deleted mutants created in this study showed significant attenuation of virulence in the *G. mellonella* killing assay. Interestingly, the virulence of DCO174 and A424 wild type was highly comparable. The reason for the observed virulence in DCO174 is not clear but *adeS*^{G178A} could be a factor contributing towards the maintenance of virulence in DCO174. In A424 derived mutant strains carrying the mutant allele *adeS*^{G178A,} the growth dynamics and biofilm forming ability was comparable to their counter-parts carrying wild type allele *adeS*^{WT}. Interestingly, the possession of *adeS*^{G178A} did not seem to be enough to reverse the *in vitro* competitive fitness associated with A424 derived strains carrying wild type allele *adeS*^{WT} as observed in the *in vitro* competitive fitness assay. It is therefore possible that the mutant DCO174 possess other, yet unknown, secondary mutations elsewhere in the genome which has severely affected the *in vitro* competitive fitness of this strain. For now, it can be said that enhanced antimicrobial resistance and virulence coupled with other secondary mutations in the genome contributed towards the highly reduced competitive fitness observed in this mutant. In future investigations, it might be possible to adopt the allele replacement approach

described this study in order to investigate whether or not the mutated efflux systems are associated with virulence in *A. baumannii*. In the next chapter I will summarize and discuss the main findings of this thesis and will provide recommendations for future work.

7 Final discussion and conclusion

The comparative sequence analysis of *A. baumannii* genome has revealed that a large proportion of the genome was occupied by the genetic materials that were acquired from various bacteria by means of horizontal gene transfer (Fournier *et al.* 2006). In 2006, Fournier *et al.* identified and described *A. baumannii* specific resistance island AbaR1 in MDR French epidemic strain AYE and a similar but empty (no resistance determinants) AbaR-like island was identified in the susceptible strains SDF. To date, several AbaR islands, numbered from AbaR0 to AbaR27, have been described in MDR *A. baumannii* strains, all of these islands have been described in MDR *A. baumannii.*

The AbaR islands are composite transposons build on the backbone of simple transposons like Tn*6019*, Tn*6021* and Tn*6022*. Towards their 5' end, these islands carry five genes *tniA, tniB, tniC, tniD and tniE* that are believed to be essential for the transposition activity. The comparative sequence analysis of the five transposition genes indicated a distant relationship of AbaR-like islands to a highly promiscuous transposon Tn*7* (Rose 2010).

The genome database was accessed from August 2014 to April 2015 in order to survey the characterized and putative AbaR islands in *A. baumannii* genome. A total of 58 unique (in terms of their host and integration site) AbaR islands were found represented in 56 *A. baumannii* strains. Most of the MDR strains carry only one copy of AbaR island in their genome, however, strains AB0057 and A85 were reported to carry two copies of AbaR-like islands in their genome.

A majority of the AbaR transposons are reported to have a backbone of transposon Tn*6019* (as in AbaR3), Tn*6021* or Tn*6022* (as in AbaR4) transposons. Few AbaR-like islands were reported to possess multiple copies of Tn*6021* and Tn*6022* making a complex looking backbone like Tn*6166*, Tn*6167* and Tn*6168*. The AbaR-like transposons that have Tn*6167*, Tn*6168* or Tn*6166* on their backbone were considered distinct from AbaR3 derived transposons and were therefore assumed to represent a separate lineage (Shaikh *et al.* 2009, Nigro and Hall 2012a). The diversification of AbaR3 islands resulting in a creation of wide range of AbaR3 variant has been extensively described Krizova *et al.* (2011). In this study, transposon backbones

represented in currently known AbaR islands were analysed for their nucleotide and amino acid similarity. The backbone transposon in GI_*comM*_A92 was named Tn*6267*.

The sequence comparison of core transposition genes of the representative backbone transposons clearly showed three distinct lineages of AbaR islands. The first lineage comprises of AbaR4-like islands. The members of this family are most abundant. They carry Tn*6022*, Tn*6021*, Tn*6166* and Tn*6167* in their backbone and usually have the *sup* gene interrupted by Tn*2006*. The novel AbaR element found in strains A92 and IS116 forms the second lineage of AbaR elements. The backbone transposon of these novel AbaR islands has been named Tn*6267* in this study. The *comM* associated AbaR islands in strains A92 and IS116 were given interim name GI_*comM*_A92 and GI_*comM*_IS116 respectively. The GI_*comM*_IS116-like islands formed a second distinct family of AbaR islands and at the present time, it appears to be only distantly related to other known AbaR elements. It is like that islands like GI_*comM*_A92 and other known AbaR elements have evolved from a common, yet unknown, ancestral transposon. The third family consists of AbaR3-like islands. The members of this family carry Tn*6019* on their backbone and usually have the *uspA* gene interrupted by large compound transposon Tn*6018*-MARR-Tn*6018*. While the AbaR3 and AbaR4 families appeared to have diverted only recently, on the other hand, the islands like GI_*comM*_IS116 might have diverted very early during their evolution from a common, yet unknown, transposon ancestor.

The AbaR-like transposons are reported as unstable features in the genome of *A. baumannii*. There are several reports of IS mediated insertion, deletion and rearrangements within these transposons (Harmer *et al.* 2014). Also, intramolecular recombination between ΔTn*6022*b and corresponding region of Tn*6022*a/Tn*2006* was reported in the AbaR25 transposon (Saule *et al.* 2013). The ability of Tn*6018* to transpose on its own was described in recent years (Williams *et al.* 2002). In this study, for the first time, I am reporting the spontaneous excision and subsequent deletion of 28.6 kb Tn*6018*-MARR segment from Tn*AbaR23* in a MDR strain A424. Following excision from its native site, the Tn*6018*-MARR segment did not re-integrate elsewhere in the genome and was lost in entirety from the cell. In the strain A424, this

spontaneous transposition was found to be occurring in *in vitro* culture condition and in the absence of antibiotic stress. In every 10^4 A424 wild type cells, around 100- 1000 cells were found to harbor the deletion of Tn*6018*-MARR region. Also, out of every 10^5 cells, around 100-1000 cells were found to possess the excised Tn*6018*-MARR as a double stranded circular entity. From A424 wild type, spontaneous mutants that have completely lost their Tn*6018*-MARR region were isolated using a protocol that ensures no prior exposure to any antibiotics. The absence of Tn*6018*-MARR region in the genome of spontaneous mutants shows that post excision, the Tn*6018*-MARR circular element does not re-integrate elsewhere in the genome and is lost in its entirety from the cell. The spontaneous deletion of Tn*6018*-MARR was also observed within AbaR1 transposon in strain AYE during this study. In both the strains A424 and AYE, the spontaneous deletion of this nature was found occurring under the laboratory culture conditions in the absence of antibiotic stress and it is therefore very likely that the spontaneous deletion of Tn*6018*-MARR is a common phenomenon which is happening across many other AbaR islands at least in *in vitro* growth conditions.

While examining the nature of spontaneous deletion within Tn*AbaR23*, it was noticed that the deletion of one copy of Tn*6018* associated transposase gene *tnpA* resulted in the reduction of deletion of Tn*6018*-MARR by 1000 folds. Interestingly, the circular Tn*6018*-MARR was not detected when only one copy of *tnpA* was knocked out. Observations from this study further supports previous research on the role of *tnpA* in mediating transposition (Williams *et al.* 2002). Although the transposition ability and target of excised Tn*6018*-MARR entity is not known at this stage, it can be hypothesized that these circular entities specifically recognize Tn*6018* and integrate via homologous recombination at the target site much like the “translocatable units” reported by Harmer *et al.* (2014). Future studies involving the experimental demonstration of transposition of Tn*6018*-MARR entity, it mode of integration and target selection would be highly commended for future studies. The spontaneous excision of Tn*6018*-MARR was observed in *in vitro* settings in this study. It would be fascinating to know whether the spontaneous deletion of this nature occurs in AbaR islands in the *in vivo* and/or environmental settings since it could be a very significant

event in terms of dissemination and acquisition of a large cargo of antimicrobial resistance determinants.

In almost all MDR strains, the MARR associated resistance determinants have been found to contribute minimally to antibiotic resistance of the host. Therefore, preservation of AbaR islands as a resistance determinant seems to be an inadequate explanation in the face of apparent instability of MARR segments within AbaR-like islands. The contribution of TnA*baR23*, an AbaR3-like island in strain A424, on the fitness and virulence phenotypes of the host was examined in this study by deleting Tn*AbaR23 en bloc.* In a head-to-head growth competition assay, the parent wild type appeared less fit compared to its Tn*AbaR23* deleted mutants and it appeared that the possession of Tn*AbaR23* was physiologically costly for the wild type strain A424 when grown in a mixed culture. However, in the *Galleria* killing assay, the Tn*AbaR23* deleted mutants displayed attenuated virulence compared to their parent wild type. Although Tn*AbaR23* did not contributed substantially towards the overall resistance phenotype of A424, preservation of this island appeared to be important for the A424 strain in order to preserve and enhance virulence-associated phenotypes.

In this study the role of AbaR-like resistance island Tn*AbaR23* was studied, for the first time, for its contribution on the fitness and virulence phenotypes of the host strain A424. The Tn*AbaR23* element and corresponding Tn*6018*-MARR segments were found to contribute positively to antibiotic resistance and virulence phenotype of the host by a yet unknown molecular mechanism. Therefore, despite being energetically costly, the preservation of Tn*AbaR23* island in its intact form appeared important for the strain in order to maintain its drug resistance and particularly virulence-associated phenotypes.

Understanding of the overall role of widespread AbaR islands could help shed light in the understanding of emergence, dissemination and persistence of highly virulent *A. baumannii* strains, therefore, the phenotypic traits associated with AbaR islands and other various genomic resistance islands in MDR *A. baumannii* strains warrants more studies. Most importantly, this work has changed the perception of “resistance

islands" solely as resistance determinants. This study therefore suggests investigating the overall phenotype associated with the foreign acquired genomic islands in order to fully appreciate their role in the persistence, virulence and survival of the bacteria.

8 Future recommendations

Further work is required to prove the possible cross talk between the Tn*AbaR23* island and virulence phenotype of the host strain. The work can be extended to other MDR *A. baumannii* strains in order to get a better picture of the link between AbaR-like island and virulence/pathogenesis of the host strain.

The instability within Tn*AbaR23* and AbaR1 has been demonstrated in this study. But there are several questions that have remained unanswered. For example:

1. Does homologous recombination between two segments of Tn*6018* play a role in the observed spontaneous deletion within the AbaR transposons?
2. What is the phenotype associated with Tn*6018*-MARR?
3. Is the observed spontaneous deletion one of mechanisms by which *A. baumannii* disseminates a cargo of genes in the environment?
4. Is it possible to capture Tn*6018*-MARR in its circular form and assess its transposition ability and virulence phenotype?
5. If the spontaneous deletion of this kind is occurring in the natural environment of *A. baumannii*, what could be the fate of the circular Tn*6018*-MARR, can this element naturally transform into other bacteria?

In order to fully understand the role played by transposase gene *tnpA* in the spontaneous deletion of Tn*6018*-MARR, the study similar to the present work can be done in the *recA* minus strains of *A. baumannii.* To rule out the requirement of intact length of Tn*6018* for homologous recombination for the spontaneous excision event in tnpA deleted mutant strains, rate of spontaneous excision of Tn*6018*-MARR can be studied in new mutant strains carrying functionally disrupted *tnpA* gene (by insertional mutagenesis) and unaltered length of Tn*6018* transposon. The complementation of Tn*6018*-MARR blocks did not help pin point the region within Tn*6018*-MARR associated with enhanced fitness.

Future work involving sequencing of spontaneous mutants and identification of virulence associated genes including the single gene knock-out/complementation in spontaneous mutant background can help better understand the mechanism involved in the enhanced virulence associated with Tn*AbaR23* and other AbaR-like islands. Also,

island deletion and phenotypic assays carried out in this study can be applied to study the contribution of large genomic islands in the virulence of pathogenic bacteria.

9 Appendices

Index

Appendix A
Nucleotide and amino acid sequence analysis

Artificial concatenated *TniA*BCDE

>*Acinetobacter baumannii* strain 1656-2

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVSN
IIKQAINDEYLNAKKPSISKTIEIVKAECSRLLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPNAD
YPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELDID
AEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRGTY
DSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEATI
QRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFEYK
QVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSDDL
WSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGDSN
MGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLMRK
YETKMLIIDEIHNCLTGSAKLLPRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVVLNGQYYWLPKTQ

>*Acinetobacter baumannii* strain 3208

MQNYINRHECLNGEFFMKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENINFLKKFRRSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPL
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FNGENTLTESLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
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NQSKKAVVSESLQTSDDLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDIL
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KAEQVLKQKFGFYNQSPVTSQVWFEIARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVL
LSILDQIMDIPCDLLVQRSNEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLN
LLRRSNNSGARHIDMERGILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGEFARLPRDDEQA
ICNECIKKQPCIRCNQTNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETC
PSCQKHRLLESDISGQRTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYT
GWLEKKVGSHKAALYINKHTHFFMKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSER
DQMEKLVQRILQPSFAYDVVLEYKNKLEEKIKRGDTSIRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQ
AAALTGFINFLNENYGISIDYLKLKKSSFLKTKQKKKLEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKI
KTEMITEIEDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain A1-clone-GCI

MQNYINRHECLNGEFFMKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENINFLKKFRRSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPL
TAETVIWVNQNDAFARNIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTV
FNGENTLTESLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
KAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMC
IASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLE

MHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERV
SDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYF
KVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQ
NQSKKAVVSESLQTSDDLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDIL
NRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFR
ASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYAS
RFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDI
VHKFKWLKPTEGLRNIRNINLSLSMRNHYRELEMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGK
WRALTIDLDKGVDAERFDALLSHSMESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLK
SRENPPYLRLMWRMGWHCSCVEHQVSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQN
KAEQVLKQKFGFYNQSPVTSQVWFEIARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVL
LSILDQIMDIPCDLLVQRSNEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLN
LLRRSNNSGARHIDMERGILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGEFARLPRDDEQA
ICNECIKKQPCIRCNQTNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETC
PSCQKHRLLESDISGQRTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYT
GWLEKKVGSHKAALYINKHTHFFMKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSER
DQMEKLVQRILQPSFAYDVVLEYKNKLEEKIKRGDTSIRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQ
AAALTGFINFLNENYGISIDYLKLKKSSFLKTKQKKKLEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKI
KTEMITEIEDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain A85

MKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTVFNGENTLTESLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQNQSKKAVVSESLQTSD
DLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMRNHYRELEMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAER
FDALLSHSMESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLKSRENPPYLRLMWRMGW
HCSCVEHQVSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQNKAEQVLKQKFGFYNQS
PVTSQVWFEIARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVLLSILDQIMDIPCDLLV
QRSNEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLNLLRRSNNSGARHIDMI
AAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYTGWLEKKVGSHKAALYINKHTHFFMKTEIDWNQSVPTP
KQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSFAYDVVLEYKNKLEEKIKRGDTS
IRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQAAALTGFINFLNENYGISIDYLKLKKSSFLKTKQKKK
LEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain A91-clone-GC2

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLR

>*Acinetobacter baumannii* strain A424

MKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTVFNGENTLTESLNKGDQ

DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQNQSKKAVVSESLQTSD
DLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAERFDALLSHSM
ESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLKSRENPPYLRLMWRMGWHCSCVEHQV
SLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQNKAEQVLKQKFGFYNQSPVTSQVWFE
IARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVLLSILDQIMDIPCDLLVQRSNEYGVS
RANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLNLLRRSNNSGARHIDMERGILTENVI
EHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGEFARLPRDDEQAICNECIKKQPCIRCNQTNKPIGKLT
EYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETCPSCQKHRLLESDISGQRTCKKCRDK
PQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYTGWLEKKVGSHKAALYINKHTHFFMK
TEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSFAYDVVLEYKN
KLEEKIKRGDTSIRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQAAALTGFINFLNENYGISIDYLKLK
KSSFLKTKQKKKLEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGYDVRFNGHSYWLPK
PIELQKNS

>*Acinetobacter baumannii* strain A9337

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSNYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLAEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHIDAVDQQLLLDTLQKLGQSTINQLPAHLFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFVR
NIMIESNAVSIDRSRITLKPNVVVGYEGNPYKIVNVLNANDIVISSLDSVRSLQVNSKSLTVFEGENTLTENLNKGDK
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLRDYRENNSLMTLIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNNYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELN
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPDDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYFKVPTAKREIPPISLFE
YRQVQNYLKNGRQDVQNQDEIYKAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMRNHYREVEMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAER
FDALLSHSMESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQVCVECLKSHENPPYLRLMWRIGW
HCSCVEHQLSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLNALNFQNKAEQVLKQKIGFYNQS
PVTTQVWFEIARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVLLSMLDQIMDIPCDLLV
QRSKEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLNLLRRSNNSGAMHID

>*Acinetobacter baumannii* strain A9380

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLR

>*Acinetobacter baumannii* strain AB0057_AbaR3

MKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTVFNGENTLTESLNKGDQ

DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQNQSKKAVVSESLQTSD
DLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAERFDALLSHSM
ESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLKSRENPPYLRLMWRMGWHCSCVEHQL
SLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQNKAEQVLKQKFGFYNQSPVTSQVWFE
IARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVLLSILDQIMDIPCDLLVQRSNEYGVS
RANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLNLLRRSNNSGARHIDMIAAGCADLCD
DCYWHQNLWNKFDQNQKVFESSYLKQQYENYTGWLEKKVGSHKAALYINKHTHFFMKTEIDWNQSVPTPKQLLVRLRS
SGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSFAYDVVLEYKNKLEEKIKRGDTSIRSARLAIK
PAVALMLSIGEESDQLPNLEHVKAYLADYSGQAAALTGFINFLNENYGISIDYLKLKKSSFLKTKQKKKLEMELVALT
QTDLSDNELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain AB0057_AbaR4

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAERFDALLSHSM
ESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQVCVECLKSHENPPYLRLMWRIGWHCSCVEHQL
SLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLNALNFQNKAEQVLKQKIGFYNQSPVTTQVWFE
IARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVLLSMLDQIMDIPCDLLVQRSKEYGVS
RANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLNLLRRSNNSGAMHIDMTENVIEHDCH
GCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGDFARLPRDDEQAICNECIKKQPCIRCNQTNKPIGKLTEYGVV
CNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETCPSCQKHRLLESDVSGQRTCKKCRDKPQKSC
KACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSDLKQQYENYIGWLEKKVGSHKAALYINKHTHFFIKTEIDW
NQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSLAYDVVLEYKNKLEEK
IKRGETSIRSARLAVKPAVALMLSMEGESAQLPNLEHVKAYLAEYSGQAAALTGFINFLNENYGASIDLFKIKKSDFL
KTKQKKKLEMELIALTQTDLNDSELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVVLNGQYYWLPKTQ

>*Acinetobacter baumannii* strain AB210

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLR

>*Acinetobacter baumannii* strain AC12

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVVLNGQYYWLPKTQ

>*Acinetobacter baumannii* strain AC29

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLR

>*Acinetobacter baumannii* strain AC30

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLP

>*Acinetobacter baumannii* strain ATCC17978

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYVGLTHYHLMVVGYE
GNPYKIVNVLNANDIVISSLDSVRSLQVNSKSLTVFEGENTLTENLNKGDKDISNEAWQIALQRYEIIKPLIEYSTTE
LVENRANEYDVNRSTLWKWLRDYRENNSLMTLIPKKRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVK
AECSRLQLEAPHENSIRRRIEALNNYQVTKARLGSKAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIESWMTSLEKR
LENLIMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIH
WEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGT
SPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKR
KFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARS
LTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSDDLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERI
ETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLP
ILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNE
LSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLG
DLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNIRNINLSLSMSQPEQCWYIRTPIQQGEIFSSWLIRSAL
DVGCSPMVLIEALWGKWRALTIDLDKGVDAERFDALLSHSMESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTR
NRSNTSGRQVCVECLKSHENPPYLRLMWRIGWHCSCVEHQLSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRR
CEESKNINLNALNFQNKAEQVLKQKIGFYNQSPVTTQVWFEIARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPV
TPLAFEYLSTQERIVLLSMLDQIMDIPCDLLVQRSKEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAIT
VTKPKSKATVQRQWLNLLRRSNNSGAMHIDMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSDLKQQYENYIGWLEK

KVGSHKAALYINKHTHFFIKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEK
LVQRILQPSLAYDVVLEYKNKLEEKIKRGETSIRSARLAVKPAVALMLSMEGESAQLPNLEHVKAYLAEYSGQAAALT
GFINFLNENYGASIDYLKLKKSDFLKTKQKKKLEMELIALTQTDLNDSELILSWVRNGLRYFHQLPYIDALKIKTEMI
TEIEDGFTVVLNGQYYWLPKTQ

>*Acinetobacter baumannii* strain AYE

MQNYINRHECLNGEFFMKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENINFLKKFRRSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPL
TAETVIWVNQNDAFARNIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTV
FNGENTLTESLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
KAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMC
IASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLE
MHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERV
SDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYF
KVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQ
NQSKKAVVSESLQTSDDLWNTPLTAFDDLRMMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDI
LNRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPF
RASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYA
SRFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYD
IVHKFKWLKPTEGLRNIRNINLSLSMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDL
DKGVDAERFDALLSHSMESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLKSRENPPYL
RLMWRMGWHCSCVEHQVSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQNKAEQVLKQ
KFGFYNQSPVTSQVWFEIARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVLLSILDQIM
DIPCDLLVQRSNEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLNLLRRSNNS
GARHIDMERGILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGEFARLPRDDEQAICNECIKK
QPCIRCNQTNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETCPSCQKHRL
LESDISGQRTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYTGWLEKKVG
SHKAALYINKHTHFFMKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQ
RILQPSFAYDVVLEYKNKLEEKIKRGDTSIRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQAAALTGFI
NFLNENYGISIDYLKLKKSSFLKTKQKKKLEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKIKTEMITEI
EDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain BJAB0868

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEYLAEHVLEIMQLSDSERIETLFTDRWIGYKKAHSIVNKTFLSFIFNGLFSC

>*Acinetobacter baumannii* strain BJAB07104

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHLFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFVR
NIMIESNAVSIDRSRITLKPNVVVGYEGNPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAERFDALLSHSM

ESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQVCVECLKSHENPPYLRLMWRIGWHCSCVEHQL
SLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLNALNFQNKAEQVLKQKIGFYNQSPVTTQVWFE
IARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVLLSMLDQIMDIPCDLLVQRSKEYGVS
RANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLNLLRRSNNSGAMHIDMTENVIEHDCH
GCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGDFARLPRDDEQAICNECIKKQPCIRCNQTNKPIGKLTEYGVV
CNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETCPSCQKHRLLESDVSGQRTCKKCRDKPQKSC
KACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSDLKQQYENYIGWLEKKVGSHKAALYINKHTHFFIKTEIDW
NQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSLAYDVVLEYKNKLEEK
IKRGETSIRSARLAVKPAVALMLSMEGESAQLPNLEHVKAYLAEYSGQAAALTGFINFLNENYGASIDYLKLKKSALY
TTNFTEPLSYQGSAFLKLPKFP

>*Acinetobacter baumannii* strain BJAB0715

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSNYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLAEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHIDAVDQQLLLDTLQKLGQSTINQLPAHLFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFVR
NIMIESNAVSIDRSRITLKPNVVVGYEGNPYKIVNVLNANDIVISSLDSVRSLQVNSKSLTVFEGENTLTENLNKGDK
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLRDYRENNSLMTLIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNNYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELN
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPDDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYFKVPTAKREIPPISLFE
YRQVQNYLKNGRQDVQNQDEIYKAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEYLAEHVLEIMQLSDSERIETLFTDRWIGYKKAHSIVNKTFLSFIFNGLFSC

>*Acinetobacter baumannii* strain D2

MQNYINRHECLNGEFFMKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENIYFLKKFRRSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPL
TAETVIWVNQNDAFARNIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTV
FNGENTLTESLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
KAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMC
IASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLE
MHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERV
SDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYF
KVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQ
NQSKKAVVSESLQTSDDLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDIL
NRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFR
ASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYAS
RFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDI
VHKFKWLKPTEGLRNIRNINLSLSMRNHYRELEMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGK
WRALTIDLDKGVDAERFDALLSHSMESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLK
SRENPPYLRLMWRMGWHCSCVEHQVSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQN
KAEQVLKQKFGFYNQSPVTSQVWFEIARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVL
LSILDQIMDIPCDLLVQRSNEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLN
LLRRSNNSGARHIDMERGILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGEFARLPRDDEQA
ICNECIKKQPCIRCNQTNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETC
PSCQKHRLLESDISGQRTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYT
GWLEKKVGSHKAALYINKHTHFFMKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSER
DQMEKLVQRILQPSFAYDVVLEYKNKLEEKIKRGDTSIRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQ
AAALTGFINFLNENYGISIDYLKLKKSSFLKTKQKKKLEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKI
KTEMITEIEDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain D13

MQNYINRHECLNGEFFMKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENINFLKKFRRSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPL
TAETVIWVNQNDAFARNIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTV
FNGENTLTESLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
KAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMC
IASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLE
MHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERV
SDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYF

KVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQ
NQSKKAVVSESLQTSDDLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDIL
NRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFR
ASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYAS
RFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDI
VHKFKWLKPTEGLRNIRNINLSLSMRNHYRELEMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGK
WRALTIDLDKGVDAERFDALLSHSMESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLK
SRENPPYLRLMWRMGWHCSCVEHQVSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQN
KAEQVLKQKFGFYNQSPVTSQVWFEIARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVL
LSILDQIMDIPCDLLVQRSNEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLN
LLRRSNNSGARHIDMERGILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGEFARLPRDDEQA
ICNECIKKQPCIRCNQTNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETC
PSCQKHRLLESDISGQRTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYT
GWLEKKVGSHKAALYINKHTHFFMKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSER
DQMEKLVQRILQPSFAYDVVLEYKNKLEEKIKRGDTSIRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQ
AAALTGFINFLNENYGISIDYLKLKKSSFLKTKQKKKLEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKI
KTEMITEIEDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain D36

MQNYINRHECLNGDFFMKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENINFLKKFRRSHVGAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPL
TAETVIWVNQNDAFARNIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTI
FNGGNTLTERLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
KAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMC
IASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLE
MHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERV
SDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYF
KVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQ
NQSKKAVVSESLQTSDDLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDIL
NRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFR
ASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYAS
RFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDI
VHKFKWLKPTEGLRNIRNINLSLSMRNHYREVEMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLIEALWGK
WRALTIDLDKGVDAERFDALLSHSMESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQVCVECLK
SHENPPYLRLMWRIGWHCSCVEHQLSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLNALNFQN
KAEQVLKQKIGFYNQSPVTTQVWFEIARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVL
LSMLDQIMDIPCDLLVQRSKEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLN
LLRRSNNSGAMHIDMERCILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGDFARLPRDDEQA
ICNECIKKQPCIRCNQTNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETC
PSCQKHRLLESDVSGQRTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSDLKQQYENYI
GWLEKKVGSHKAALYINKHTHFFIKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSER
DQMEKLVQRILQPSLAYDVVLEYKNKLEEKIKRGETSIRSARLAVKPAVALMLSMEGESAQLPNLEHVKAYLAEYSGQ
AAALTGFINFLNENYGASIDYLKLKKSDFLKTKQKKKLEMELIALTQTDLNDSELILSWVRNGLRYFHQLPYIDALKI
KTEMITEIEDGFTVVLNGQYYWLPKTQ

>*Acinetobacter baumannii* strain D81-clone-GC1

MQNYINRHECLNGEFFMKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENINFLKKFRRSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPL
TAETVIWVNQNDAFARNIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTV
FNGENTLTESLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
KAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMC
IASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLE
MHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERV
SDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYF
KVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQ
NQSKKAVVSESLQTSDDLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDIL
NRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFR
ASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYAS
RFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDI
VHKFKWLKPTEGLRNIRNINLSLSMRNHYRELEMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGK
WRALTIDLDKGVDAERFDALLSHSMESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLK
SRENPPYLRLMWRMGWHCSCVEHQVSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQN

KAEQVLKQKFGFYNQSPVTSQVWFEIARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVL
LSILDQIMDIPCDLLVQRSNEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLN
LLRRSNNSGARHIDMERGILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGEFARLPRDDEQA
ICNECIKKQPCIRCNQTNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETC
PSCQKHRLLESDISGQRTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYT
GWLEKKVGSHKAALYINKHTHFFMKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSER
DQMEKLVQRILQPSFAYDVVLEYKNKLEEKIKRGDTSIRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQ
AAALTGFINFLNENYGISIDYLKLKKSSFLKTKQKKKLEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKI
KTEMITEIEDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain IOMTU 433

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAERFDALLSHSM
ESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQVCVECLKSHENPPYLRLMWRIGWHCSCVEHQL
SLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLNALNFQNKAEQVLKQKIGFYNQSPVTTQVWFE
IARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVLLSMLDQIMDIPCDLLVQRSKEYGVS
RANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLNLLRRSNNSGAMHIDMIAAGCADLCD
DCYWHQNLWNKFDQNQKVFESSDLKQQYENYIGWLEKKVGSHKAALYINKHTHFFIKTEIDWNQSVPTPKQLLVRLRS
SGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSLAYDVVLEYKNKLEEKIKRGETSIRSARLAVK
PAVALMLSMEGESAQLPNLEHVKAYLAEYSGQAAALTGFINFLNENYGASIDYLKLKKSDFLKTKQKKKLEMELIALT
QTDLNDSELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVVLNGQYYWLPKTQ

>*Acinetobacter baumannii* strain IS116

MYNQLCDTVKIVPARRKVGNTRISVSGLYPFKKDSAVAFESTLERDFLIRLEIDPNVLAVESQPFTIEYIDNGKKRVY
TPDFLVTYKHDSYLPFIPPKLVEVKPAAELEQNLNLWKTRYRAAMIICKEEGWKFHIAHEGRIRDRLWLNAMFLQKYR
KMNFSFADSKHLIDYVRDREVVTFDTLLARHFSGKWDRANGISQIWYLVAHGYLVCDLSLPLQPSTELWISGNMNDRR
INIKIAAGESVQVKGVPHTILEIIDLKSVFVENKNTGRKGIVAISELQPALLTEKYEDIDLIPDKDWAEAERRFNIIR
PFIEDGVSGRKEVELRAKQHDINPATLYRWLKIYNAYGTIDGLRPKKEGLPQGQKMINKHAEAIIEKAIKDLYLTNQR
ISVQKVVLEVKRLCHEYHILDVPHANTVRNRIAAISQREVLRKRGHKEKANNRFTPAAGKFPHADYPLAVVQIDHTPV
DLILVDDFNRQPIGRPYLTLAIDVFSRVVVGYYLSLDAPSVMSIAMCVSQAVLPKEKWLALRKIQAEWPVWGIMNTIH
VDNGPEFHSETFRNACMAHDIRLEYRPVKKPRFGGHIERLMGTFATDIHAVPGTTFSNIFQRKDYDSDTQAVFTFTEF
EDWLIDFICNVYHKRKHSALGTSPLHAFELGIFGDKNTLGSGAARLPANDHNFFLDFLPSFERTIQTTGVTIDGLAYY
ADVLRSWINSIDPDNHKEKRKFIFRRDPRNISEIWFFDPEIKNYFKVPLADQSIPPFSIWEHKKIQELKKETGEYLKD
HELGQALAKLRERVQEASIKTRRARRAYQRSTQHQQSINPASIQDKKSQQESTVMEQQLENLFGLLPLDELENDEDIS
MSNYPHIFQEFRPIVDQSNEDRLYFLEEDRWIGYPAANDLLDQLKGMLTLPKRSRMPNLLVLSKPNNGKTSIINQFFK
LYGEGYINELNNAVKPVIVVQAPVSPDEKALYMAILDRFWEPFRERDPVAKLRYQVVHCLKLYEVKLLIIDEMNSLLC
GTPIRQRTVMNAIKYLCNETHIPVVGFGTEEAVRVLYTDPQHVSRFRVVNLPLWQLDKEFQALVNKFEKVLPLKQPSR
LAEPATVKYLHDITEGNLGNLRSLLREAAKKAIISGQEYIDHHLLEDVRMAPTRETYYWVTRTPLLQDEALSSWLIRV
ALGCGCDPLSLTGVIWPKWRVWTIDIDKGLTQEHLEILARKTAVSQDQLKNATFKKLFLQNSAINLEPWILALGTRNR
KHKSGWQYCPKCLESDPVAYFRLNWRYVLHVGCVKHNQRLLDQCPHCQKAIQPRLLEAPDQTLSCCALCKEKLFDVKA
DVIDHNALALQKDFDLFLKQGYAIYNDTLIPISEWLSVIQLFNQFIRKVLRSSLNSKGWAFLNALDISVPHPQLSSTG
LVLSQISVLEREKIFACISQLLKVSQKKFIETAHSLSMNRASFWDKRCKIPEILIPLEKQLDKVSRGYILKGNVSSTP
RPTCKKAILRKMWRLKRKILMPQCDDCGRSVEKIHKNYKSTKFCHTCYVRVFKKRACSSCGKLARLYKYDNSAICQKC
ENNRPCIRCQRVDYSIGKITKYGPVCCSCSVYFKEFQACERCGCFSQKLSRISRFSDNLRVCPKCATRDYRTCPSCRR
YRLLEEDVKSGQMYCKKCLNSPPHYCLICKLKIPAGRGNYCESCSWHQILERRVGKLANNLVDTHLRKHFKNYIKWLE
QRVGSHKAALFTAKHIKFFEETEDLWIEQVPAYTELLGRLRTSGLRKFVLPMQWLTQVHHLQVDIQAKEFCSELDQLN
RLKNICLEPTFPAQILQKYFDTLMNRVSDGTTTIRSARLAMKPASALMFLVSNSRFNLPRIWHVKHYLSHHPGQAAEL
IGFIIFLNRNYDTNLNFSFIKNSNFIKAIKNQKLEKEIIKLSKLTKNRFELLLWVRLCLMYFHKFEITHSKQIELNMI
NEIEDGLEISFRNEIFWIPKINNFFDISQEST

>*Acinetobacter baumannii* strain K51-65

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLR

>*Acinetobacter baumannii* strain K51-74

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLR

>*Acinetobacter baumannii* strain LT-3

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLR

>*Acinetobacter baumannii* strain LT-11

MTHLNELYLILNKSLKWNKSHLKCFALIMLVIILKQTCNLSSASKALPIKCLPQSFYRRMQRFFAGQYFDYRQISQLI
FNMFSFDQVQLTLDRTNWKWGKRNINILMLAIVYRGIAIPILWTLLNKRGNSDTKERIALIQRFIAIFGKDRIVNVFA
DREFIGEQWFTWLIEQDINFCIRVKKTSLSPIIMVGRVKLYISALQLENGELLLVVSPQFNANAIQDYALRWEIETLF
SCLKGRGFNLGNTRLTDPRRVKKLIAVLAISFCWCYLTGEWQHDQKKAIKIKKHGRLSMSLFRYGLDYVQMAIQRLIG
FGKKEEFKEILAILRRQNPDRIRVL

>*Acinetobacter baumannii* strain LT-V1

TQLSEQNQSKKAVVSESLQTSDDLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMN
TLTDILNRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNH
FFVPFRASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHT
DPQYASRFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDE
EITYDIVHKFKWLKPTEGLRNIRNINLSLSMRNHYREVEMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLI
EALWGKWRALTIDLDKGVDAERFDALLSHSMESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQV
CVECLKSHENPPYLRLMWRIGWHCSCVEHQLSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLN
ALNFQNKAEQVLKQKIGFYNQSPVTTQVWFEIARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLST
QERIVLLSMLDQIMDIPCDLLVQRSKEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATV
QRQWLNLLRRSNNSGAMHIDMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSDLKQQYENYIGWLEKKVGSHKAALY
INKHTHFFIKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSL
AYDVVLEYKNKLEEKIKRGETSIRSARLAVKPAVALMLSMEGESAQLPNLEHVKAYLAEYSGQAAALTGFINFLNENY
GASIDLFKIKKSDFLKTKQKKKLEMELIALTQTDLNDSELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVV
LNGQYYWLPKTQ

>*Acinetobacter baumannii* strain MDR-TJ

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAERFDALLSHSM
ESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQVCVECLKSHENPPYLRLMWRIGWHCSCVEHQL
SLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLNALNFQNKAEQVLKQKIGFYNQSPVTTQVWFE
IARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVLLSMLDQIMDIPCDLLVQRSKEYGVS
RANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLNLLRRSNNSGAMHIDMTENVIEHDCH
GCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGDFARLPRDDEQAICNECIKKQPCIRCNQTNKPIGKLTEYGVV
CNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETCPSCQKHRLLESDVSGQRTCKKCRDKPQKSC
KACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSDLKQQYENYIGWLEKKVGSHKAALYINKHTHFFIKTEIDW
NQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSLAYDVVLEYKNKLEEK
IKRGETSIRSARLAVKPAVALMLSMEGESAQLPNLEHVKAYLAEYSGQAAALTGFINFLNENYGASIDYLKLKKSDFL
KTKQKKKLEMELIALTQTDLNDSELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVVLNGQYYWLPKTQ

>*Acinetobacter baumannii* strain MDR-ZJ06

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAERFDALLSHSM
ESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQVCVECLKSHENPPYLRLMWRIGWHCSCVEHQL
SLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLNALNFQNKAEQVLKQKIGFYNQSPVTTQVWFE
IARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVLLSMLDQIMDIPCDLLVQRSKEYGVS
RANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLNLLRRSNNSGAMHIDMTENVIEHDCH
GCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGDFARLPRDDEQAICNECIKKQPCIRCNQTNKPIGKLTEYGVV
CNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETCPSCQKHRLLESDVSGQRTCKKCRDKPQKSC
KACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSDLKQQYENYIGWLEKKVGSHKAALYINKHTHFFIKTEIDW
NQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSLAYDVVLEYKNKLEEK
IKRGETSIRSARLAVKPAVALMLSMEGESAQLPNLEHVKAYLAEYSGQAAALTGFINFLNENYGASIDYLKLKKSDFL
KTKQKKKLEMELIALTQTDLNDSELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVVLNGQYYWLPKTQ

>*Acinetobacter baumannii* strain NCGM 237

MQNYINRHECLNGDFFMKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENINFLKKFRRSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPL
TAETVIWVNQNDAFARNIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTI
FNGGNTLTERLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
KAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMC
IASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLE

MHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERV
SDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYF
KVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQ
NQSKKAVVSESLQTSDDLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDIL
NRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFR
ASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVVLNGQ
YYWLPKTQ

>*Acinetobacter baumannii* strain pA85-3

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYASRFDVVNLPKWELNQDF
LRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDIVHKFKWLKPTEGLRNI
RNINLSLSMRNHYREVEMSQPEQCWYIRTPIQQGEIFSSWLIRSALDVGCSPMVLIEALWGKWRALTIDLDKGVDAER
FDALLSHSMESKQKIQQSMLSSVVSQIQPNYDSNQNIPWVLSLGTRNRSNTSGRQVCVECLKSHENPPYLRLMWRIGW
HCSCVEHQLSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRRCEESKNINLNALNFQNKAEQVLKQKIGFYNQS
PVTTQVWFEIARSWLSEIRFLVNTPNKNVIQLFESFDVNLHLSHPVTPLAFEYLSTQERIVLLSILDQIMDIPCDLLV
QRSKEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRAAITVTKPKSKATVQRQWLNLLRRSNNSGAMHIDME
RCILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGDFARLPRDDEQAICNECIKKQPCIRCNQ
TNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISPFNDDLRVCPKCATRDYETCPSCQKHRLLESDVSGQ
RTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSDLKQQYENYIGWLEKKVGSHKAALYI
NKHTHFFIKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSERDQMEKLVQRILQPSLA
YDVVLEYKNKLEEKIKRGETSIRSARLAVKPAVALMLSMEGESAQLPNLEHVKAYLAEYSGQAAALTGFINFLNENYG
ASIDYLKLKKSDFLKTKQKKKLEMELIALTQTDLNDSELILSWVRNGLRYFHQLPYIDALKIKTEMITEIEDGFTVVL
NGQYYWLPKTQ

>*Acinetobacter baumannii* strain RUH134

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLR

>*Acinetobacter baumannii* strain RUH875

MQNYINRHECLNGEFFMKKLSDEYSPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVVGQPVV
IPYITELGNQSTYTPDFLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETR
LYDQYWENINFLKKFRRSHVDAVDQQLLLDTLQKLGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMVTCDLYCPL
TAETVIWVNQNDAFARNIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTV
FNGENTLTESLNKGDQDISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPK
KRGWTTEKSRLSPQVSNIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGS
KAAIDKFKAAAGSFPNADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMC
IASCILSKKRKLIELDIDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLE
MHVLDGTTFSNVQQRGTYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERV
SDEDTLFIDFLPEFEATIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPFSNTYF
KVPTAKREIPPISLFEYKQVQNYLKSERQDVQNQDEIYMAILHLREQLNQARSLTRKQRRSNQRKKENEKAITQLSEQ
NQSKKAVVSESLQTSDDLWNTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDIL
NRPRKLRPECLLIVGDSNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFR

ASDPEAKLRNQAVHLMRKYETKMLIIDEIHNCLTGSAKLLPEVMNTLKNLSNELSLNIVGVGTREAITILHTDPQYAS
RFDVVNLPKWELNQDFLRLLVSYVRLLPLKKQSNLASKEIATLIFEVSGGNLGDLNRLLVECAKEAILQGDEEITYDI
VHKFKWLKPTEGLRNIRNINLSLSMRNHYRELEMSQPEQCWYIRTPIQQGEVFSSWLIRSALDVGCSPMVLIEALWGK
WRALTIDLDKGVDAERFDALLSHSMESKQNIQQSMLSSVVSEIHTNYDPKQNTPWVLSLGTRNRSNTSGRQVCVECLK
SRENPPYLRLMWRMGWHCSCVEHQVSLIDHCPECGVTIQPFKADMEHGCLAICTTCGFDLRHCEENKIINLNALDFQN
KAEQVLKQKFGFYNQSPVTSQVWFEIARAWLSEIRFLVNTPNKNVIQLFESFDVNLHLPNPITPLAFEYLNTQERIVL
LSILDQIMDIPCDLLVQRSNEYGVSRANFWDKRKKLPVQLQQMKDLMIKPTRRYPVSRVAITVTKPKSKATVQRQWLN
LLRRSNNSGARHIDMERGILTENVIEHDCHGCNQSVSFIKKRYKGKKYCSTCYARIFKKRLCPSCGEFARLPRDDEQA
ICNECIKKQPCIRCNQTNKPIGKLTEYGVVCNSCSVYFRPIEPCERCGTPSQKLTRISRFNDDLRVCPKCATRDYETC
PSCQKHRLLESDISGQRTCKKCRDKPQKSCKACHCMIAAGCADLCDDCYWHQNLWNKFDQNQKVFESSYLKQQYENYT
GWLEKKVGSHKAALYINKHTHFFMKTEIDWNQSVPTPKQLLVRLRSSGLRKFELVMQWLEEVHDIRIDMDNKKSCSER
DQMEKLVQRILQPSFAYDVVLEYKNKLEEKIKRGDTSIRSARLAIKPAVALMLSIGEESDQLPNLEHVKAYLADYSGQ
AAALTGFINFLNENYGISIDYLKLKKSSFLKTKQKKKLEMELVALTQTDLSDNELILSWVRNGLRYFHQLPYIDALKI
KTEMITEIEDGYDVRFNGHSYWLPKPIELQKNS

>*Acinetobacter baumannii* strain TCDC-AB0715

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
ADYPLAYVQIDHTPLDIEIVDDEFREAIGKPHLTLAIDVFSRMIVGYYLSLEAPSTTSVAMCIASCILSKKRKLIELD
IDAEWQVEGIMDSVHTDNGPDFRTNHISKACLKYGIHWEYRPIGGARFGGHIERMLGIVNLEMHVLDGTTFSNVQQRG
TYDSAKQACMTLKELEYYIVYWITKVYHQKKHSALGTSPIVKWEEGVWGTKTTAGTGLKERVSDEDTLFIDFLPEFEA
TIQRTGVQKDNLFYFADCLRQWVNSIDPEDNNRKRKRKFLFKRDPRDISQIWFYEPYSNTYFKVPTAKREIPPISLFE
YKQVQNYLKSERQDVQNQDEIYKAILHLRDQLNQARSLTRKQRRSNQRKKENAKAITQLSEQNQSKKAVVSESLQTSD
DLWSTPLTAFDDLRMEKYEHLAEHVLEIMQLSDSERIETLFTDRWIGYKKAITIMNTLTDILNRPRKLRPECLLIVGD
SNMGKTTIIHEFARQYYTKTVSDADMDLLSVTKPVLPILAPAKANVKELYINILNHFFVPFRASDPEAKLRNQAVHLM
RKYETKMLIIDEIHNCLTGSAKLLPQKWA

>*Acinetobacter baumannii* strain TYTH-1

MKKLSDEYLPVRKAQTVYGSISGNYAFRGEKTIWFESTLERDFILKQEFNNNVIDVIGQPVVIPYITELGNQSTYTPD
FLVQFSSSNCDDFEDFPVPMLIEIKPKKKLVEDWDKLKPKFRAAHRFAADKGWKFKIISETRLYDQYWENINFLKKFR
RSHVGAVDQQLLLDTLQELGQSTINQLPAHIFKDKTNVLKGIHQVWALVAKRMIACDLYCPLTAETVIWVNQNDAFAR
NIMIESNAVNIDRSRIILKPNVVVGYEGQPYKIVNVLNANDIVISSLDSVRSLQVNARSLTIFNGGNTLTERLNKGDQ
DISNEAWQIALQRYEIIKPLIEYSTTELVENRANEYDVNRSTLWKWLKDYRENNSLMALIPKKRGWTTEKSRLSPQVS
NIIKQAINDEYLNAKKPSISKTIEIVKAECSRLQLEAPHENSIRRRIEALNDYQVTKARLGSKAAIDKFKAAAGSFPN
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>*Acinetobacter baumannii* strain WM98

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The sequence of 3' *comM* in A92 strain showing underlined GI_*comM*_A92 left inverted repeat and <u>bold underlined</u> 3' *comM* remnant.

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Sequence of the circular junction of Tn*6018*-MARR

The primers PR2520 and PR2521 were used to sequence the 3.7 kb PCR amplicon. The following sequence is a reconstructed sequence showing **<u>bold and underlined</u>**.Tn*6018* inverted repeat sequences.

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Appendix B

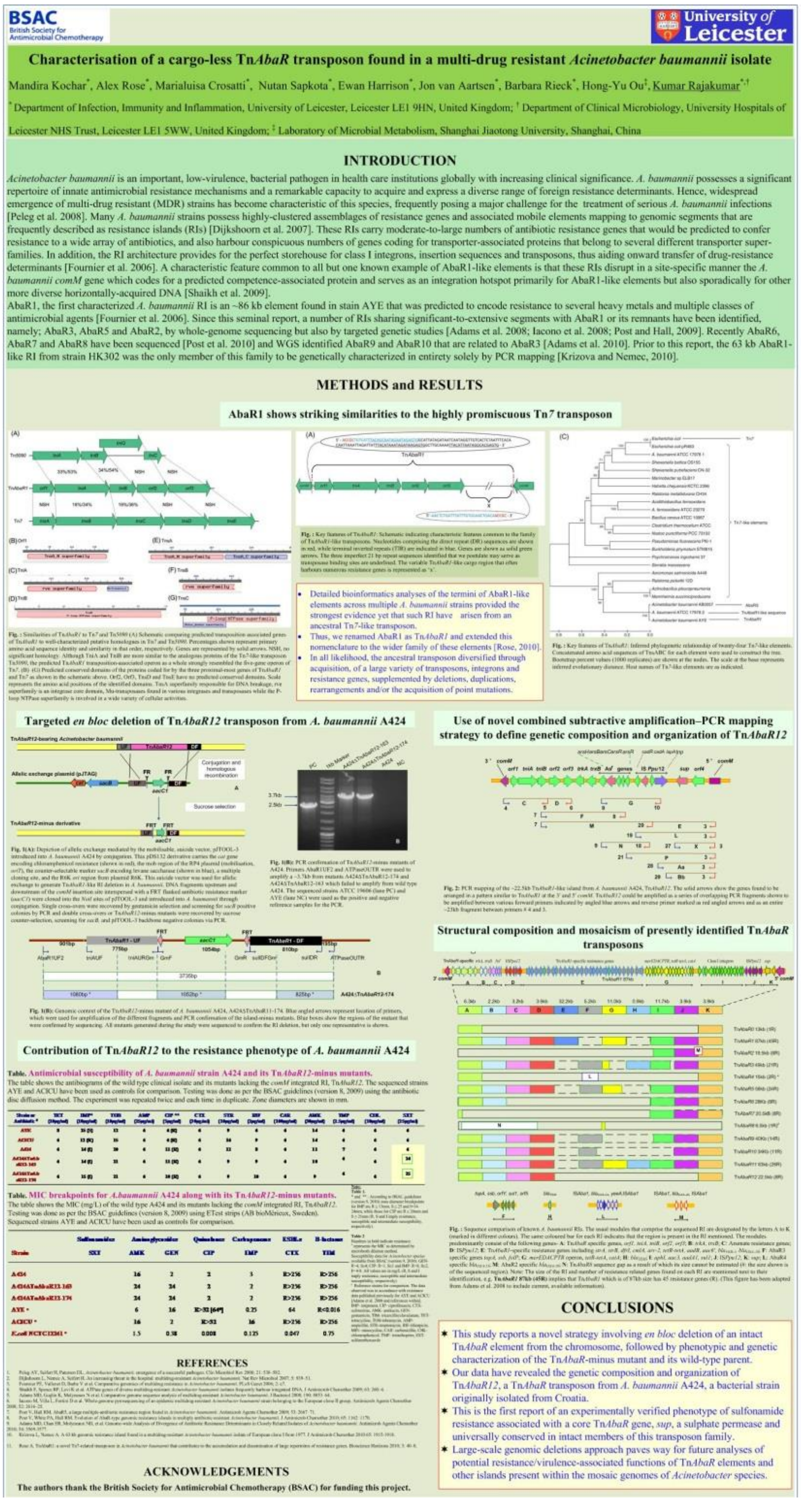
Table: Pairwise alignment of nucleotide and amino acid sequence from AbaR islands of various *A. baumannii* strains

***Acinetobacter baumannii* strains**	**IS116**	**AB0057_AbaR3**	**A424**	**BJAB0715**	**ATCC17978**	**IOMTU 433**	**AB0057_AbaR4**	**BJAB0868**	**A91-clone-GC2**	**RUH134**
IS116		52	53	52	52	52	52	52	52	52
AB0057_AbaR3	38		100	93	96	96	96	96	97	97
A424	40	100		93	95	96	96	96	97	97
BJAB0715	39	95	95		95	93	93	95	93	93
ATCC17978	38	95	95	94		99	98	96	98	98
IOMTU 433	38	97	97	95	98		100	98	100	100
AB0057_AbaR4	40	97	97	95	98	100		98	100	100
BJAB0868	39	97	97	97	94	98	98		99	99
A91-clone-GC2	40	98	98	97	96	100	100	100		100
RUH134	40	98	98	97	96	100	100	100	100	

A stretch of 6763 bp nucleotide sequence from artificially concatenated *tniABCDE* gene and 2095 amino acid long sequence of artificially concatenated TniABCDE protein were aligned by pairwise alignment to obtain the percentage identity. Values in shaded area show the percentage similarity of the TniABCDE protein from AbaR elements of various representative *A. baumannii* strains used in this study. Values in non-shaded area are the percentage identity of nucleotide between the *tniABCDE* genes of various AbaR elements from representative *A. baumannii* strains.

Appendix C

Posters presented in various events



DOES EFFLUX SYSTEM UP-REGULATION ACCOUNT FOR THE UNUSUAL CIPROFLOXACIN RESISTANCE PHENOTYPE OBSERVED IN A TARGETED *ACINETOBACTER BAUMANNII* MUTANT OBTAINED FOLLOWING GENTAMICIN EXPOSURE?

University of Leicester

M. Crosatti, C. Holmes, J. Chan, N. Sapkota, M. Pallen, K. Rajakumar

1. Observation of an unusual phenotype

In Kochar *et al.*, we described the unusual antibiotic resistance profile of one of two mutants created by deleting Tn*AbaR23* genomic island. This island was predicted to confer antibiotic resistance to sulfamethoxazole but not ciprofloxacin:

Strain	Characteristic	Sulfamethoxazole MIC	Ciprofloxacin MIC
A424	WT	>1024µg/ml	4µg/ml
DCO163	Deletion of Tn*AbaR23*	0.75µg/ml	3µg/ml
DCO174	Deletion of Tn*AbaR23*	4µg/ml	>32µg/ml

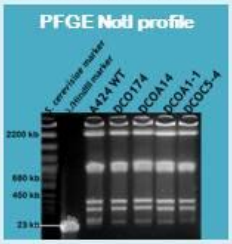
DCO163 and DCO174 Pulse Field Gel Electrophoresis (PFGE) profiles showed that were both related to A424 WT but they were different from each other.

2. New deletion mutant (DCOC5-4) does not display the unusual ciprofloxacin phenotype of DCO174

DCOC5-4 showed PFGE profile when digested with NotI undistinguishable to DCO174 but very different level of resistance to ciprofloxacin.

Strain	Ciprofloxacin (mm)
A424	16 ± 0
DCO163	16 ± 1
DCO174	6 ± 0
DCOC5-4	16 ± 1

Disc susceptibility test
Average ± standard deviation from three independent experiments



3. The efflux-pump inhibitor NMP affects resistance to ciprofloxacin of DCO174

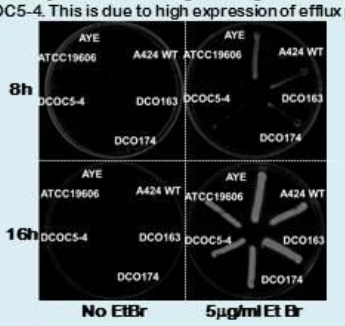
The involvement of efflux pumps was tested by measuring the variation in ciprofloxacin resistance in presence of 1-(1-naphthylmethyl)-piperazin (NMP), an RND family inhibitor. Disc susceptibility tests were performed by plating strains on Muller-Hinton agar (MHA) with and without NMP and calculating the difference in diameter.

Strain	Sulfamethoxazole (mm)	Ciprofloxacin (mm)
A424	0 ± 0	-2 ± 0
DCO163	0 ± 0	-3 ± 1
DCO174	2 ± 2	7 ± 1
DCOC5-4	0 ± 1	-2 ± 2
AYE	0 ± 1	0 ± 0
ATCC 19606	0 ± 0	1 ± 1

Average ± standard deviation from three independent experiments
Difference= (∅ on MHA+NMP) – (∅ on MHA)
For difference >0 → efflux pump is involved in resistance

4. DCO174 is extruding Ethidium bromide at 8h

When plated on Ethidium bromide, DCO174 was able to expel it more efficiently therefore looking less bright than A424, DCO163 and DCOC5-4. This is due to high expression of efflux pumps.

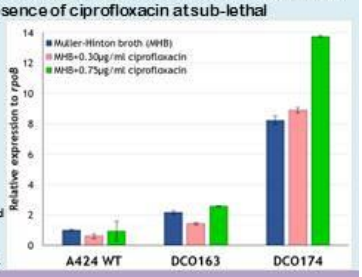


5. Genome-wide sequence comparison reveals an SNP in *adeS* of DCO174

Position	ORF	A424	DCO163	DCO174
569612	*rpoA* (RNA polymerase α)	C	A	C
2137257	*lon* (ATP-dependent protease)	C	T	C
2601753	*adeS* (two-component regulator of *adeABC*)	G	G	A
2789313	A424_2985 (TonB family protein)	G	A	G
2905881	A424_3118 (*lacRSUA* operon repressor)	G	T	G
3078118	Outside coding region	C	A	C
3256193	*metG* (methionyl-tRNA synthase)	A	C	A
3291034	*gltA* (citrate synthase I)	G	A	G

6. qRT-PCR indicates overexpression of *adeB* in DCO174

The two-component regulator *adeRS* controls the expression of *adeABC*. DCO174 showed increased expression of *adeB* at basal level compared to A424 and DCO163. The expression was further increased by the presence of ciprofloxacin at sub-lethal concentration.



Expression of *adeB* was normalized against the expression of *rpoB*. RNA samples were analyzed for integrity using the Agilent 2100 Bioanalyzer. PCRs were run in triplicate.

For more information, please write to Marialuisa Crosatti at mc444@le.ac.uk.

University of Leicester

Fitness and virulence-associated phenotypes of the plastic Tn*AbaR23* *Acinetobacter baumannii* A424 resistance mega-transposon

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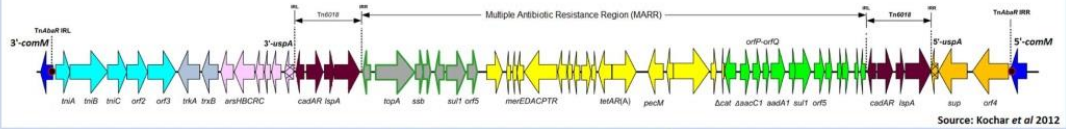
Background

The multi-drug resistant *Acinetobacter baumannii* strain A424 isolated from a human wound infection has a 48.3 kb resistance mega-transposon, Tn*AbaR23*, inserted in a site- and orientation-specific manner within the chromosomal *comM* gene. Within Tn*AbaR*23 there is a 25 kb, approximately centrally-located region which has been termed the multiple antibiotic resistance region (MARR). This MARR segment, variant versions of which are found in the vast majority of other known Tn*AbaR* mega-transposons, is bounded precisely by two directly-oriented copies of the transposon Tn*6018*. The intervening segment of the Tn*AbaR23* MARR and corresponding counterparts of other Tn*AbaR* transposons are 'packed' with antibiotic resistance determinants, heavy metal resistance determinants and efflux pump genes. We have observed the spontaneous excision and subsequent loss of the Tn*AbaR23* MARR in the strain A424. The present study aimed to further explore the phenotypic contribution of this transposon to its host strain.

Tn*AbaR23* mega-transposon 48.3 kb



Source: Kochar *et al* 2012

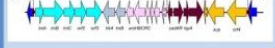
Method

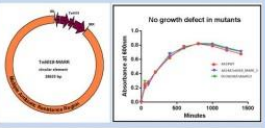
- Creation of a markerless Tn*AbaR*23-minus mutant via *en bloc* deletion by allelic exchange
- Isolation and characterization of spontaneous mutants that have lost Tn*6018*-MARR from the Tn*AbaR*23 mega-transposon
- Assessing and comparing the fitness of parent wild type strain, Tn*AbaR*23 deleted mutant and Tn*6018*-MARR deleted mutant in *in vitro* batch-based head-to-head growth competition assays
- Comparing the virulence of wild type and mutants using a larval-stage greater wax moth *Galleria mellonella* model

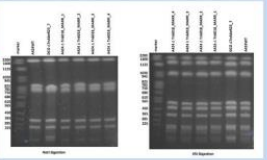
Result

- Tn*AbaR*23 island deleted *en bloc* and marker less mutant DCOA14ΔTn*AbaR*23_7 created to use in the fitness and virulence test assays
- Semi-quantitative PCR assay and colony patching assay indicated the excision and loss of Tn*6018*-MARR occurred at a frequency of approximately 1/100 cells
- Tn*6018*-MARR was found to be excising from Tn*AbaR*23 as a circular element and was being lost from the cell in entirety creating a 19.7 kb truncated version of Tn*AbaR*23 mega transposon
- Four independent versions of Tn*6018*-MARR deleted mutants were isolated and were shown by PFGE to be indistinguishable and to possess the predicted PFGE profile (A424ΔTn*6018*-MARR_3 was used in fitness and virulence assays)
- Growth curve shows no growth defect in the mutants upon the deletion of Tn*AbaR*23 either partially or *en bloc*
- DCOA14ΔTn*AbaR*23_7 and A424ΔTn*6018*-MARR_3 were subtly but significantly 'fitter' than their parent in *in vitro* head-to-head competition assay
- DCOA14_7 and A424ΔTn*6018*-MARR_3 exhibited greatly reduced virulence compared to their parent in the *Galleria* model.

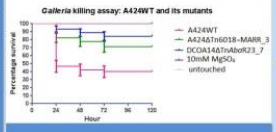
Tn*AbaR23*ΔTn*6018*-MARR 19.7 kb



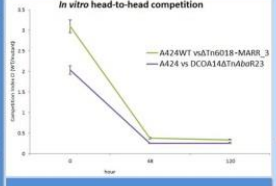




***Galleria* killing assay: A424WT and its mutants**



***In vitro* head-to-head competition**



Conclusion

Although possession of the Tn*AbaR23* resistance mega-transposon appears to be energetically and/or physiologically costly for A424, preservation of this island in its intact form seems to be important for the strain in order to maintain its drug resistance and virulence-associated phenotypes.

Upon the loss of Tn*6018*-MARR, there was a 'reversion' to sulphamethoxazole susceptibility and a reduced tetracycline MIC. As neither of these classes of antibiotic are presently frequently used to treat serious *A. baumannii* infections and as several other Tn*AbaR* MARR regions based on gene content would similarly be predicted to contribute minimally to antibiotic resistance of the cognate host strain, preservation of resistance to these agents alone would seem to be an inadequate explanation for the seeming stability of Tn*AbaR* MARR segments.

Thus in conclusion, we hypothesize that Tn*AbaR* elements and corresponding MARR segments-specifically contribute positively to antibiotic resistance and other yet-to-be molecularly-defined fitness, virulence and/or environmental survival traits that ensure the propagation and stability of these elements.

References:

- Kochar M, Crosatti M *et al.* 2012. Deletion of Tn*AbaR*23 results in both expected and unexpected antibiogram changes in a multidrug-resistant *Acinetobacter baumannii* strain. *Antimicrobial Agents and Chemotherapy*. 56(4): 1845-1853
- Rose A. 2010. Tn*AbaR*1: a novel Tn7-related transposon in *Acinetobacter baumannii* that contributes to the accumulation and dissemination of large repertoires of resistance genes. Bioscience Horizons.

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References

Abbas, S., Ahmed, I., Kudo, T., Iida, T., Ali, G.M., Fujiwara, T., and Ohkuma (2014) 'Heavy metal-tolerant and psychrotolerant bacterium Acinetobacter pakistanensis sp. nov. isolated from a textile dyeing wastewater treatment pond', *Pak. J. Agri. Sci*, 51, 595-608.

Adams, M. D., Chan, E. R., Molyneaux, N. D. and Bonomo, R. A. (2010) 'Genomewide analysis of divergence of antibiotic resistance determinants in closely related isolates of Acinetobacter baumannii', *Antimicrob Agents Chemother*, 54(9), 3569-77.

Adams, M. D., Goglin, K., Molyneaux, N., Hujer, K. M., Lavender, H., Jamison, J. J., MacDonald, I. J., Martin, K. M., Russo, T., Campagnari, A. A., Hujer, A. M., Bonomo, R. A. and Gill, S. R. (2008) 'Comparative genome sequence analysis of multidrug-resistant Acinetobacter baumannii', *J Bacteriol*, 190(24), 8053-64.

Alvarez-Perez, S., Lievens, B., Jacquemyn, H. and Herrera, C. M. (2013) 'Acinetobacter nectaris sp. nov. and Acinetobacter boissieri sp. nov., isolated from floral nectar of wild Mediterranean insect-pollinated plants', *Int J Syst Evol Microbiol*, 63(Pt 4), 1532-9.

Andersson, D. I. and Hughes, D. (2010) 'Antibiotic resistance and its cost: is it possible to reverse resistance?', *Nat Rev Microbiol*, 8(4), 260-71.

Batirel, A., Balkan, II, Karabay, O., Agalar, C., Akalin, S., Alici, O., Alp, E., Altay, F. A., Altin, N., Arslan, F., Aslan, T., Bekiroglu, N., Cesur, S., Celik, A. D., Dogan, M., Durdu, B., Duygu, F., Engin, A., Engin, D. O., Gonen, I., Guclu, E., Guven, T., Hatipoglu, C. A., Hosoglu, S., Karahocagil, M. K., Kilic, A. U., Ormen, B., Ozdemir, D., Ozer, S., Oztoprak, N., Sezak, N., Turhan, V., Turker, N. and Yilmaz, H. (2014) 'Comparison of colistin-carbapenem, colistin-sulbactam, and colistin plus other antibacterial agents for the treatment of extremely drug-resistant Acinetobacter baumannii bloodstream infections', *Eur J Clin Microbiol Infect Dis*, 33(8), 1311-22.

Baumann, P. (1968) 'Isolation of Acinetobacter from soil and water', *J Bacteriol*, 96(1), 39-42.

Baumann, P., Doudoroff, M. and Stanier, R. Y. (1968) 'A study of the Moraxella group. II. Oxidative-negative species (genus Acinetobacter)', *J Bacteriol*, 95(5), 1520-41.

Beceiro, A., Moreno, A., Fernandez, N., Vallejo, J. A., Aranda, J., Adler, B., Harper, M., Boyce, J. D. and Bou, G. (2014) 'Biological cost of different mechanisms of colistin resistance and their impact on virulence in Acinetobacter baumannii', *Antimicrob Agents Chemother*, 58(1), 518-26.

Bergogne-Berezin, E. and Towner, K. J. (1996) 'Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features', *Clin Microbiol Rev*, 9(2), 148-65.

Berlau, J., Aucken, H., Malnick, H. and Pitt, T. (1999) 'Distribution of Acinetobacter species on skin of healthy humans', *Eur J Clin Microbiol Infect Dis*, 18(3), 179-83.

Bouvet, P. J. and Jeanjean, S. (1989) 'Delineation of new proteolytic genomic species in the genus Acinetobacter', *Res Microbiol*, 140(4-5), 291-9.

Bouvet, P. J. M., and Grimont, P.A.D (1986) 'Taxonomy of the genus Acinetobacter with the recognition of Acinetobacter baumannii sp. nov., Acinetobacter haemolyticus sp. nov.,

Acinetobacter johnsonii sp. nov., and Acinetobacter junii sp. nov. and emended descriptions of Acinetobacter calcoaceticus and Acinetobacter lwoffii', *Int. J. Syst. Bacteriol*, 36, 228-240.

Brisou, J. and Prevot, A. R. (1954) '[Studies on bacterial taxonomy. X. The revision of species under Acromobacter group]', *Ann Inst Pasteur (Paris)*, 86(6), 722-8.

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S. and Snyder, M. (2010) 'Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing', *PLoS Pathog*, 6(4), e1000834.

Carr, E. L., Kampfer, P., Patel, B. K., Gurtler, V. and Seviour, R. J. (2003) 'Seven novel species of Acinetobacter isolated from activated sludge', *Int J Syst Evol Microbiol*, 53(Pt 4), 953-63.

CDC (2004) 'Acinetobacter baumannii infections among patients at military medical facilities treating injured U.S. service members, 2002-2004', *MMWR Morb Mortal Wkly Rep*, 53(45), 1063-6.

Cerqueira, G. M. and Peleg, A. Y. (2011) 'Insights into Acinetobacter baumannii pathogenicity', *IUBMB Life*, 63(12), 1055-60.

Charles, E. (1968) 'Herellea (Acinetobacter) and Pseudomonas ovalis
(P. putida) from Frozen Foods', *Applied Microbiology*, 17(No.1), 26-30.

Chaudhary, H. J., Peng, G., Hu, M., He, Y., Yang, L., Luo, Y. and Tan, Z. (2012) 'Genetic diversity of endophytic diazotrophs of the wild rice, Oryza alta and identification of the new diazotroph, Acinetobacter oryzae sp. nov', *Microb Ecol*, 63(4), 813-21.

Chaveroche, M. K., Ghigo, J. M. and d'Enfert, C. (2000) 'A rapid method for efficient gene replacement in the filamentous fungus Aspergillus nidulans', *Nucleic Acids Res*, 28(22), E97.

Choi, J. Y., Ko, G., Jheong, W., Huys, G., Seifert, H., Dijkshoorn, L. and Ko, K. S. (2013) 'Acinetobacter kookii sp. nov., isolated from soil', *Int J Syst Evol Microbiol*, 63(Pt 12), 4402-6.

Choi, K. H. and Schweizer, H. P. (2005) 'An improved method for rapid generation of unmarked Pseudomonas aeruginosa deletion mutants', *BMC Microbiol*, 5, 30.

Choi, K. Y., Li, Y., Sarnovsky, R. and Craig, N. L. (2013) 'Direct interaction between the TnsA and TnsB subunits controls the heteromeric Tn7 transposase', *Proc Natl Acad Sci U S A*, 110(22), E2038-45.

CLSI (2009) 'Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically', *Approved standard*, 29 No. 2(Eighth edition), M07-A8.

Costerton, J. W. (1999) 'Bacterial Biofilms: A Common Cause of Persistent Infections', *Science*, 284(5418), 1318-1322.

Craig, N. L. (1997) 'Target site selection in transposition', *Annu Rev Biochem*, 66, 437-74.

Dennis, J. J. and Zylstra, G. J. (1998) 'Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes', *Appl Environ Microbiol*, 64(7), 2710-5.

Di Cello, F., Pepi, M., Baldi, F. and Fani, R. (1997) 'Molecular characterization of an n-alkane-degrading bacterial community and identification of a new species, Acinetobacter venetianus', *Res Microbiol*, 148(3), 237-49.

Di Nocera, P. P., Rocco, F., Giannouli, M., Triassi, M. and Zarrilli, R. (2011) 'Genome organization of epidemic Acinetobacter baumannii strains', *BMC Microbiol*, 11, 224.

Diancourt, L., Passet, V., Nemec, A., Dijkshoorn, L. and Brisse, S. (2010) 'The population structure of Acinetobacter baumannii: expanding multiresistant clones from an ancestral susceptible genetic pool', *PLoS One*, 5(4), e10034.

Dijkshoorn, L., Nemec, A. and Seifert, H. (2007) 'An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii', *Nat Rev Microbiol*, 5(12), 939-51.

Domingues, S., Harms, K., Fricke, W. F., Johnsen, P. J., da Silva, G. J. and Nielsen, K. M. (2012) 'Natural transformation facilitates transfer of transposons, integrons and gene cassettes between bacterial species', *PLoS Pathog*, 8(8), e1002837.

Dower, W. J., Miller, J. F. and Ragsdale, C. W. (1988) 'High efficiency transformation of E. coli by high voltage electroporation', *Nucleic Acids Res*, 16(13), 6127-45.

Falagas, M. E., Bliziotis, I. A. and Siempos, II (2006) 'Attributable mortality of Acinetobacter baumannii infections in critically ill patients: a systematic review of matched cohort and case-control studies', *Crit Care*, 10(2), R48.

Feng, G. D., Yang, S. Z., Wang, Y. H., Deng, M. R. and Zhu, H. H. (2014) 'Acinetobacter guangdongensis sp. nov., isolated from abandoned lead-zinc ore', *Int J Syst Evol Microbiol*, 64(Pt 10), 3417-21.

Fernandez, A., Perez, A., Ayala, J. A., Mallo, S., Rumbo-Feal, S., Tomas, M., Poza, M. and Bou, G. (2012) 'Expression of OXA-type and SFO-1 beta-lactamases induces changes in peptidoglycan composition and affects bacterial fitness', *Antimicrob Agents Chemother*, 56(4), 1877-84.

Field, D., Wilson, G. and van der Gast, C. (2006) 'How do we compare hundreds of bacterial genomes?', *Curr Opin Microbiol*, 9(5), 499-504.

Fournier, P. E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., Richet, H., Robert, C., Mangenot, S., Abergel, C., Nordmann, P., Weissenbach, J., Raoult, D. and Claverie, J. M. (2006) 'Comparative genomics of multidrug resistance in Acinetobacter baumannii', *PLoS Genet*, 2(1), e7.

Frost, L. S., Leplae, R., Summers, A. O. and Toussaint, A. (2005) 'Mobile genetic elements: the agents of open source evolution', *Nat Rev Microbiol*, 3(9), 722-32.

Gaddy, J. A. and Actis, L. A. (2009) 'Regulation of Acinetobacter baumannii biofilm formation', *Future Microbiol*, 4(3), 273-8.

Gan, H. M., Lean, S. S., Suhaili, Z., Thong, K. L. and Yeo, C. C. (2012) 'Genome sequence of Acinetobacter baumannii AC12, a polymyxin-resistant strain isolated from Terengganu, Malaysia', *J Bacteriol*, 194(21), 5979-80.

Gao, F., Wang, Y., Liu, Y. J., Wu, X. M., Lv, X., Gan, Y. R., Song, S. D. and Huang, H. (2011) 'Genome sequence of Acinetobacter baumannii MDR-TJ', *J Bacteriol*, 193(9), 2365-6.

Gaynes, R. and Edwards, J. R. (2005) 'Overview of nosocomial infections caused by gram-negative bacilli', *Clin Infect Dis*, 41(6), 848-54.

Gerner-Smidt, P., Tjernberg, I. and Ursing, J. (1991) 'Reliability of phenotypic tests for identification of Acinetobacter species', *J Clin Microbiol*, 29(2), 277-82.

Gottig, S., Gruber, T. M., Higgins, P. G., Wachsmuth, M., Seifert, H. and Kempf, V. A. (2014) 'Detection of pan drug-resistant Acinetobacter baumannii in Germany', *J Antimicrob Chemother*, 69(9), 2578-9.

Guo, B., Abdelraouf, K., Ledesma, K. R., Nikolaou, M. and Tam, V. H. (2012) 'Predicting bacterial fitness cost associated with drug resistance', *J Antimicrob Chemother*, 67(4), 928-32.

Guzman, L. M., Belin, D., Carson, M. J. and Beckwith, J. (1995) 'Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter', *J Bacteriol*, 177(14), 4121-30.

Hamidian, M. and Hall, R. M. (2011) 'AbaR4 replaces AbaR3 in a carbapenem-resistant Acinetobacter baumannii isolate belonging to global clone 1 from an Australian hospital', *J Antimicrob Chemother*, 66(11), 2484-91.

Hamidian, M., Hancock, D. P. and Hall, R. M. (2013) 'Horizontal transfer of an ISAba125-activated ampC gene between Acinetobacter baumannii strains leading to cephalosporin resistance', *J Antimicrob Chemother*, 68(1), 244-5.

Hamidian, M., Kenyon, J. J., Holt, K. E., Pickard, D. and Hall, R. M. (2014a) 'A conjugative plasmid carrying the carbapenem resistance gene blaOXA-23 in AbaR4 in an extensively resistant GC1 Acinetobacter baumannii isolate', *J Antimicrob Chemother*, 69(10), 2625-8.

Hamidian, M., Wynn, M., Holt, K. E., Pickard, D., Dougan, G. and Hall, R. M. (2014b) 'Identification of a marker for two lineages within the GC1 clone of Acinetobacter baumannii', *J Antimicrob Chemother*, 69(2), 557-8.

Hanahan, D., Jessee, J. and Bloom, F. R. (1991) 'Plasmid transformation of Escherichia coli and other bacteria', *Methods Enzymol*, 204, 63-113.

Harmer, C. J., Moran, R. A. and Hall, R. M. (2014) 'Movement of IS26-associated antibiotic resistance genes occurs via a translocatable unit that includes a single IS26 and preferentially inserts adjacent to another IS26', *MBio*, 5(5), e01801-14.

Harrison, E. M., Carter, M. E., Luck, S., Ou, H. Y., He, X., Deng, Z., O'Callaghan, C., Kadioglu, A. and Rajakumar, K. (2010) 'Pathogenicity islands PAPI-1 and PAPI-2 contribute individually and synergistically to the virulence of Pseudomonas aeruginosa strain PA14', *Infect Immun*, 78(4), 1437-46.

Hornsey, M., Loman, N., Wareham, D. W., Ellington, M. J., Pallen, M. J., Turton, J. F., Underwood, A., Gaulton, T., Thomas, C. P., Doumith, M., Livermore, D. M. and Woodford, N. (2011) 'Whole-genome comparison of two Acinetobacter baumannii isolates from a single patient, where resistance developed during tigecycline therapy', *J Antimicrob Chemother*, 66(7), 1499-503.

Iacono, M., Villa, L., Fortini, D., Bordoni, R., Imperi, F., Bonnal, R. J., Sicheritz-Ponten, T., De Bellis, G., Visca, P., Cassone, A. and Carattoli, A. (2008) 'Whole-genome pyrosequencing of an epidemic multidrug-resistant Acinetobacter baumannii strain belonging to the European clone II group', *Antimicrob Agents Chemother*, 52(7), 2616-25.

Imperi, F., Antunes, L. C., Blom, J., Villa, L., Iacono, M., Visca, P. and Carattoli, A. (2011) 'The genomics of Acinetobacter baumannii: insights into genome plasticity, antimicrobial resistance and pathogenicity', *IUBMB Life*, 63(12), 1068-74.

Isberg, R. R., Iwashkiw, J. A., Seper, A., Weber, B. S., Scott, N. E., Vinogradov, E., Stratilo, C., Reiz, B., Cordwell, S. J., Whittal, R., Schild, S. and Feldman, M. F. (2012) 'Identification of a General O-linked Protein Glycosylation System in Acinetobacter baumannii and Its Role in Virulence and Biofilm Formation', *PLoS Pathogens*, 8(6), e1002758.

Joseph, P. and Craig, N. L. (2001) 'Tn7 smarter than we thought ', *Nature Reviews*, 2.

Jung, J. and Park, W. (2015) 'Acinetobacter species as model microorganisms in environmental microbiology: current state and perspectives', *Appl Microbiol Biotechnol*, 99(6), 2533-48.

Kang, Y. S., Jung, J., Jeon, C. O. and Park, W. (2011) 'Acinetobacter oleivorans sp. nov. is capable of adhering to and growing on diesel-oil', *J Microbiol*, 49(1), 29-34.

Kempf, M. and Rolain, J. M. (2012) 'Emergence of resistance to carbapenems in Acinetobacter baumannii in Europe: clinical impact and therapeutic options', *Int J Antimicrob Agents*, 39(2), 105-14.

Kenyon, J. J. and Hall, R. M. (2013) 'Variation in the complex carbohydrate biosynthesis loci of Acinetobacter baumannii genomes', *PLoS One*, 8(4), e62160.

Kim, D., Baik, K. S., Kim, M. S., Park, S. C., Kim, S. S., Rhee, M. S., Kwak, Y. S. and Seong, C. N. (2008) 'Acinetobacter soli sp. nov., isolated from forest soil', *J Microbiol*, 46(4), 396-401.

Kim, D. H. and Ko, K. S. (2015) 'AbaR-type genomic islands in non-baumannii Acinetobacter species isolates from South Korea', *Antimicrob Agents Chemother*, 59(9), 5824-6.

Kim, P. S., Shin, N. R., Kim, J. Y., Yun, J. H., Hyun, D. W. and Bae, J. W. (2014) 'Acinetobacter apis sp. nov., isolated from the intestinal tract of a honey bee, Apis mellifera', *J Microbiol*, 52(8), 639-45.

Kochar, M., Crosatti, M., Harrison, E. M., Rieck, B., Chan, J., Constantinidou, C., Pallen, M., Ou, H. Y. and Rajakumar, K. (2012) 'Deletion of TnAbaR23 results in both expected and

unexpected antibiogram changes in a multidrug-resistant Acinetobacter baumannii strain', *Antimicrob Agents Chemother*, 56(4), 1845-53.

Krizova, L., Dijkshoorn, L. and Nemec, A. (2011) 'Diversity and evolution of AbaR genomic resistance islands in Acinetobacter baumannii strains of European clone I', *Antimicrob Agents Chemother*, 55(7), 3201-6.

Krizova, L., Maixnerova, M., Sedo, O. and Nemec, A. (2014) 'Acinetobacter bohemicus sp. nov. widespread in natural soil and water ecosystems in the Czech Republic', *Syst Appl Microbiol*, 37(7), 467-73.

Krizova, L., McGinnis, J., Maixnerova, M., Nemec, M., Poirel, L., Mingle, L., Sedo, O., Wolfgang, W. and Nemec, A. (2015) 'Acinetobacter variabilis sp. nov. (formerly DNA group 15 sensu Tjernberg & Ursing), isolated from humans and animals', *Int J Syst Evol Microbiol*, 65(Pt 3), 857-63.

Krizova, L. and Nemec, A. (2010) 'A 63 kb genomic resistance island found in a multidrug-resistant Acinetobacter baumannii isolate of European clone I from 1977', *J Antimicrob Chemother*, 65(9), 1915-8.

Lee, H. J. and Lee, S. S. (2010) 'Acinetobacter kyonggiensis sp. nov., a beta-glucosidase-producing bacterium, isolated from sewage treatment plant', *J Microbiol*, 48(6), 754-9.

Lee, J. S., Lee, K. C., Kim, K. K., Hwang, I. C., Jang, C., Kim, N. G., Yeo, W. H., Kim, B. S., Yu, Y. M. and Ahn, J. S. (2009) 'Acinetobacter antiviralis sp. nov., from Tobacco plant roots', *J Microbiol Biotechnol*, 19(3), 250-6.

Li, W., Zhang, D., Huang, X. and Qin, W. (2014) 'Acinetobacter harbinensis sp. nov., isolated from river water', *Int J Syst Evol Microbiol*, 64(Pt 5), 1507-13.

Li, Y., He, W., Wang, T., Piao, C. G., Guo, L. M., Chang, J. P., Guo, M. W. and Xie, S. J. (2014) 'Acinetobacter qingfengensis sp. nov., isolated from canker bark of Populus x euramericana', *Int J Syst Evol Microbiol*, 64(Pt 3), 1043-50.

Li, Y., Piao, C. G., Ma, Y. C., He, W., Wang, H. M., Chang, J. P., Guo, L. M., Wang, X. Z., Xie, S. J. and Guo, M. W. (2013) 'Acinetobacter puyangensis sp. nov., isolated from the healthy and diseased part of Populus xeuramericana canker bark', *Int J Syst Evol Microbiol*, 63(Pt 8), 2963-9.

Liou, M. L., Liu, C. C., Lu, C. W., Hsieh, M. F., Chang, K. C., Kuo, H. Y., Lee, C. C., Chang, C. T., Yang, C. Y. and Tang, C. Y. (2012) 'Genome sequence of Acinetobacter baumannii TYTH-1', *J Bacteriol*, 194(24), 6974.

LiveFood, U. [online], available: http://www.livefood.co.uk [accessed

Lockhart, S. R., Abramson, M. A., Beekmann, S. E., Gallagher, G., Riedel, S., Diekema, D. J., Quinn, J. P. and Doern, G. V. (2007) 'Antimicrobial resistance among Gram-negative bacilli causing infections in intensive care unit patients in the United States between 1993 and 2004', *J Clin Microbiol*, 45(10), 3352-9.

Longo, B., Pantosti, A., Luzzi, I., Placanica, P., Gallo, S., Tarasi, A., Di Sora, F., Monaco, M., Dionisi, A. M., Volpe, I., Montella, F., Cassone, A. and Rezza, G. (2006) 'An outbreak of

Acinetobacter baumannii in an intensive care unit: epidemiological and molecular findings', *J Hosp Infect*, 64(3), 303-5.

Lopez-Rojas, R., Dominguez-Herrera, J., McConnell, M. J., Docobo-Perez, F., Smani, Y., Fernandez-Reyes, M., Rivas, L. and Pachon, J. (2011) 'Impaired Virulence and In Vivo Fitness of Colistin-Resistant Acinetobacter baumannii', *Journal of Infectious Diseases*, 203(4), 545-548.

MacKenzie, F. M., Struelens, M. J., Towner, K. J. and Gould, I. M. (2005) 'Report of the Consensus Conference on Antibiotic Resistance; Prevention and Control (ARPAC)', *Clin Microbiol Infect*, 11(11), 938-54.

Mak, J. K., Kim, M. J., Pham, J., Tapsall, J. and White, P. A. (2009) 'Antibiotic resistance determinants in nosocomial strains of multidrug-resistant Acinetobacter baumannii', *J Antimicrob Chemother*, 63(1), 47-54.

Malhotra, J., Anand, S., Jindal, S., Rajagopal, R. and Lal, R. (2012) 'Acinetobacter indicus sp. nov., isolated from a hexachlorocyclohexane dump site', *Int J Syst Evol Microbiol*, 62(Pt 12), 2883-90.

Malloy, J. L., Veldhuizen, R. A., Thibodeaux, B. A., O'Callaghan, R. J. and Wright, J. R. (2005) 'Pseudomonas aeruginosa protease IV degrades surfactant proteins and inhibits surfactant host defense and biophysical functions', *Am J Physiol Lung Cell Mol Physiol*, 288(2), L409-18.

Maragakis, L. L. and Perl, T. M. (2008) 'Acinetobacter baumannii: epidemiology, antimicrobial resistance, and treatment options', *Clin Infect Dis*, 46(8), 1254-63.

Metzgar, D., Bacher, J. M., Pezo, V., Reader, J., Doring, V., Schimmel, P., Marliere, P. and de Crecy-Lagard, V. (2004) 'Acinetobacter sp. ADP1: an ideal model organism for genetic analysis and genome engineering', *Nucleic Acids Res*, 32(19), 5780-90.

Miyata, S., Casey, M., Frank, D. W., Ausubel, F. M. and Drenkard, E. (2003) 'Use of the Galleria mellonella caterpillar as a model host to study the role of the type III secretion system in Pseudomonas aeruginosa pathogenesis', *Infect Immun*, 71(5), 2404-13.

Mullany, P., Allan, E. and Roberts, A. P. (2015) 'Mobile genetic elements in Clostridium difficile and their role in genome function', *Res Microbiol*, 166(4), 361-7.

Nemec, A., De Baere, T., Tjernberg, I., Vaneechoutte, M., van der Reijden, T. J. and Dijkshoorn, L. (2001) 'Acinetobacter ursingii sp. nov. and Acinetobacter schindleri sp. nov., isolated from human clinical specimens', *Int J Syst Evol Microbiol*, 51(Pt 5), 1891-9.

Nemec, A., Dijkshoorn, L., Cleenwerck, I., De Baere, T., Janssens, D., Van Der Reijden, T. J., Jezek, P. and Vaneechoutte, M. (2003) 'Acinetobacter parvus sp. nov., a small-colony-forming species isolated from human clinical specimens', *Int J Syst Evol Microbiol*, 53(Pt 5), 1563-7.

Nemec, A., Krizova, L., Maixnerova, M., Sedo, O., Brisse, S. and Higgins, P. G. (2015) 'Acinetobacter seifertii sp. nov., a member of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex isolated from human clinical specimens', *Int J Syst Evol Microbiol*, 65(Pt 3), 934-42.

Nemec, A., Krizova, L., Maixnerova, M., van der Reijden, T. J., Deschaght, P., Passet, V., Vaneechoutte, M., Brisse, S. and Dijkshoorn, L. (2011) 'Genotypic and phenotypic characterization of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex with the proposal of Acinetobacter pittii sp. nov. (formerly Acinetobacter genomic species 3) and Acinetobacter nosocomialis sp. nov. (formerly Acinetobacter genomic species 13TU)', *Res Microbiol*, 162(4), 393-404.

Nemec, A., Musilek, M., Maixnerova, M., De Baere, T., van der Reijden, T. J., Vaneechoutte, M. and Dijkshoorn, L. (2009) 'Acinetobacter beijerinckii sp. nov. and Acinetobacter gyllenbergii sp. nov., haemolytic organisms isolated from humans', *Int J Syst Evol Microbiol*, 59(Pt 1), 118-24.

Nemec, A., Musilek, M., Sedo, O., De Baere, T., Maixnerova, M., van der Reijden, T. J., Zdrahal, Z., Vaneechoutte, M. and Dijkshoorn, L. (2010) 'Acinetobacter bereziniae sp. nov. and Acinetobacter guillouiae sp. nov., to accommodate Acinetobacter genomic species 10 and 11, respectively', *Int J Syst Evol Microbiol*, 60(Pt 4), 896-903.

Nigro, S. J. and Hall, R. M. (2012a) 'Antibiotic resistance islands in A320 (RUH134), the reference strain for Acinetobacter baumannii global clone 2', *J Antimicrob Chemother*, 67(2), 335-8.

Nigro, S. J. and Hall, R. M. (2012b) 'Tn6167, an antibiotic resistance island in an Australian carbapenem-resistant Acinetobacter baumannii GC2, ST92 isolate', *J Antimicrob Chemother*, 67(6), 1342-6.

Nigro, S. J., Post, V. and Hall, R. M. (2011) 'The multiresistant Acinetobacter baumannii European clone I type strain RUH875 (A297) carries a genomic antibiotic resistance island AbaR21, plasmid pRAY and a cluster containing ISAba1-sul2-CR2-strB-strA', *J Antimicrob Chemother*, 66(8), 1928-30.

Nishimura Y, T. I., and H Iizuka (1988) 'Acinetobacter radioresistens sp. nov. Isolated from Cotton and Soil', *International Journal of Systematic Bacteriology*, 38(2), 209-211.

Ozdem, B., Gurelik, F. C., Celikbilek, N., Balikci, H. and Acikgoz, Z. C. (2011) '[Antibiotic resistance profiles of Acinetobacter species isolated from several clinical samples between 2007-2010]', *Mikrobiyol Bul*, 45(3), 526-34.

Parks, A. R. and Peters, J. E. (2009) 'Tn7 elements: engendering diversity from chromosomes to episomes', *Plasmid*, 61(1), 1-14.

Peleg, A. Y., Seifert, H. and Paterson, D. L. (2008) 'Acinetobacter baumannii: emergence of a successful pathogen', *Clin Microbiol Rev*, 21(3), 538-82.

Poirel, L. and Nordmann, P. (2006) 'Carbapenem resistance in Acinetobacter baumannii: mechanisms and epidemiology', *Clin Microbiol Infect*, 12(9), 826-36.

Post, V. and Hall, R. M. (2009) 'AbaR5, a large multiple-antibiotic resistance region found in Acinetobacter baumannii', *Antimicrob Agents Chemother*, 53(6), 2667-71.

Post, V., Hamidian, M. and Hall, R. M. (2012) 'Antibiotic-resistant Acinetobacter baumannii variants belonging to global clone 1', *J Antimicrob Chemother*, 67(4), 1039-40.

Post, V., White, P. A. and Hall, R. M. (2010) 'Evolution of AbaR-type genomic resistance islands in multiply antibiotic-resistant Acinetobacter baumannii', *J Antimicrob Chemother*, 65(6), 1162-70.

Rieck, B., Tourigny, D. S., Crosatti, M., Schmid, R., Kochar, M., Harrison, E. M., Ou, H. Y., Turton, J. F. and Rajakumar, K. (2012) 'Acinetobacter insertion sequence ISAba11 belongs to a novel family that encodes transposases with a signature HHEK motif', *Appl Environ Microbiol*, 78(2), 471-80.

Roberts, A. P. and Kreth, J. (2014) 'The impact of horizontal gene transfer on the adaptive ability of the human oral microbiome', *Front Cell Infect Microbiol*, 4, 124.

Roberts, J., Holcenberg, J. S. and Dolowy, W. C. (1972) 'Isolation, crystallization, and properties of Achromobacteraceae glutaminase-asparaginase with antitumor activity', *J Biol Chem*, 247(1), 84-90.

Rose, A. (2010) 'TnAbaR1: a novel Tn7-related transposon in Acinetobacter baumannii that contributes to the accumulation and dissemination of large repertoires of resistance genes', *Bioscience Horizons*, 3, 40-48.

Saranathan, R., Tomar, A., Sudhakar, P., Arunkumar, K. P. and Prashanth, K. (2014) 'Draft Genome Sequence of a Multidrug-Resistant Acinetobacter baumannii PKAB07 Clinical Strain from India Belonging to Sequence Type 195', *Genome Announc*, 2(2).

Saule, M., Samuelsen, O., Dumpis, U., Sundsfjord, A., Karlsone, A., Balode, A., Miklasevics, E. and Karah, N. (2013) 'Dissemination of a carbapenem-resistant Acinetobacter baumannii strain belonging to international clone II/sequence type 2 and harboring a novel AbaR4-like resistance island in Latvia', *Antimicrob Agents Chemother*, 57(2), 1069-72.

Seputiene, V., Povilonis, J. and Suziedeliene, E. (2012) 'Novel variants of AbaR resistance islands with a common backbone in Acinetobacter baumannii isolates of European clone II', *Antimicrob Agents Chemother*, 56(4), 1969-73.

Shaikh, F., Spence, R. P., Levi, K., Ou, H. Y., Deng, Z., Towner, K. J. and Rajakumar, K. (2009) 'ATPase genes of diverse multidrug-resistant Acinetobacter baumannii isolates frequently harbour integrated DNA', *J Antimicrob Chemother*, 63(2), 260-4.

Smani, Y., Lopez-Rojas, R., Dominguez-Herrera, J., Docobo-Perez, F., Marti, S., Vila, J. and Pachon, J. (2012) 'In vitro and in vivo reduced fitness and virulence in ciprofloxacin-resistant Acinetobacter baumannii', *Clin Microbiol Infect*, 18(1), E1-4.

Smet, A., Cools, P., Krizova, L., Maixnerova, M., Sedo, O., Haesebrouck, F., Kempf, M., Nemec, A. and Vaneechoutte, M. (2014) 'Acinetobacter gandensis sp. nov. isolated from horse and cattle', *Int J Syst Evol Microbiol*, 64(Pt 12), 4007-15.

Smith, M. G., Gianoulis, T. A., Pukatzki, S., Mekalanos, J. J., Ornston, L. N., Gerstein, M. and Snyder, M. (2007) 'New insights into Acinetobacter baumannii pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis', *Genes Dev*, 21(5), 601-14.

Starikova, I., Al-Haroni, M., Werner, G., Roberts, A. P., Sorum, V., Nielsen, K. M. and Johnsen, P. J. (2013) 'Fitness costs of various mobile genetic elements in Enterococcus faecium and Enterococcus faecalis', *J Antimicrob Chemother*, 68(12), 2755-65.

Tada, T., Miyoshi-Akiyama, T., Shimada, K., Shimojima, M. and Kirikae, T. (2014) 'Dissemination of 16S rRNA methylase ArmA-producing acinetobacter baumannii and emergence of OXA-72 carbapenemase coproducers in Japan', *Antimicrob Agents Chemother*, 58(5), 2916-20.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S. (2013) 'MEGA6: Molecular Evolutionary Genetics Analysis version 6.0', *Mol Biol Evol*, 30(12), 2725-9.

Teo, J., Lim, T. P., Hsu, L. Y., Tan, T. Y., Sasikala, S., Hon, P. Y., Kwa, A. L. and Apisarnthanarak, A. (2015) 'Extensively drug-resistant Acinetobacter baumannii in a Thai hospital: a molecular epidemiologic analysis and identification of bactericidal Polymyxin B-based combinations', *Antimicrob Resist Infect Control*, 4(1), 2.

Tjernberg, I. and Ursing, J. (1989) 'Clinical strains of Acinetobacter classified by DNA-DNA hybridization', *Apmis*, 97(7), 595-605.

Valenzuela, J. K., Thomas, L., Partridge, S. R., van der Reijden, T., Dijkshoorn, L. and Iredell, J. (2007) 'Horizontal gene transfer in a polyclonal outbreak of carbapenem-resistant Acinetobacter baumannii', *J Clin Microbiol*, 45(2), 453-60.

Vallenet, D., Nordmann, P., Barbe, V., Poirel, L., Mangenot, S., Bataille, E., Dossat, C., Gas, S., Kreimeyer, A., Lenoble, P., Oztas, S., Poulain, J., Segurens, B., Robert, C., Abergel, C., Claverie, J. M., Raoult, D., Medigue, C., Weissenbach, J. and Cruveiller, S. (2008) 'Comparative analysis of Acinetobacters: three genomes for three lifestyles', *PLoS One*, 3(3), e1805.

van Hoek, A. H., Mevius, D., Guerra, B., Mullany, P., Roberts, A. P. and Aarts, H. J. (2011) 'Acquired antibiotic resistance genes: an overview', *Front Microbiol*, 2, 203.

Vaneechoutte, M., De Baere, T., Nemec, A., Musilek, M., van der Reijden, T. J. and Dijkshoorn, L. (2008) 'Reclassification of Acinetobacter grimontii Carr et al. 2003 as a later synonym of Acinetobacter junii Bouvet and Grimont 1986', *Int J Syst Evol Microbiol*, 58(Pt 4), 937-40.

Vaz-Moreira, I., Novo, A., Hantsis-Zacharov, E., Lopes, A. R., Gomila, M., Nunes, O. C., Manaia, C. M. and Halpern, M. (2011) 'Acinetobacter rudis sp. nov., isolated from raw milk and raw wastewater', *Int J Syst Evol Microbiol*, 61(Pt 12), 2837-43.

Viehman, J. A., Nguyen, M. H. and Doi, Y. (2014) 'Treatment options for carbapenem-resistant and extensively drug-resistant Acinetobacter baumannii infections', *Drugs*, 74(12), 1315-33.

Vila, J., Marti, S. and Sanchez-Cespedes, J. (2007) 'Porins, efflux pumps and multidrug resistance in Acinetobacter baumannii', *J Antimicrob Chemother*, 59(6), 1210-5.

Waddell, C. S. and Craig, N. L. (1989) 'Tn7 transposition: Recognition of the attTn7 target sequence', *Proc. Natl. Acad. Sci. USA*, 86(June), 3958-62.

Williams, P. A., Jones, R. M. and Shaw, L. E. (2002) 'A Third Transposable Element, ISPpu12, from the Toluene-Xylene Catabolic Plasmid pWW0 of Pseudomonas putida mt-2', *J Bacteriol*, 184(23), 6572-6580.

Wootton, M. (2013) 'BSAC Methods for Antimicrobial Susceptibility Testing', Version 12.

Yoon, E. J., Courvalin, P. and Grillot-Courvalin, C. (2013) 'RND-type efflux pumps in multidrug-resistant clinical isolates of Acinetobacter baumannii: major role for AdeABC overexpression and AdeRS mutations', *Antimicrob Agents Chemother*, 57(7), 2989-95.

Yoon, J. H., Kim, I. G. and Oh, T. K. (2007) 'Acinetobacter marinus sp. nov. and Acinetobacter seohaensis sp. nov., isolated from sea water of the Yellow Sea in Korea', *J Microbiol Biotechnol*, 17(11), 1743-50.

Zhou, H., Zhang, T., Yu, D., Pi, B., Yang, Q., Zhou, J., Hu, S. and Yu, Y. (2011) 'Genomic analysis of the multidrug-resistant Acinetobacter baumannii strain MDR-ZJ06 widely spread in China', *Antimicrob Agents Chemother*, 55(10), 4506-12.

Zhu, L., Yan, Z., Zhang, Z., Zhou, Q., Zhou, J., Wakeland, E. K., Fang, X., Xuan, Z., Shen, D. and Li, Q. Z. (2013) 'Complete genome analysis of three Acinetobacter baumannii clinical isolates in China for insight into the diversification of drug resistance elements', *PLoS One*, 8(6), e66584.